PROGRAMMING DENDRITIC CELLS FOR THE 'KICK AND KILL' OF LATENT HIV-1

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Submitted to the Graduate Faculty of

Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2019

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

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It was defended on

April 24, 2019

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ABSTRACT

Finding a nontoxic, effective means to purge the latent HIV-1 reservoir in virally suppressed individuals undergoing antiretroviral therapy (ART) remains a major obstacle to a functional cure. The 'kick and kill' approach to controlling HIV-1 involves induction of HIV-1 latency reversal (LR) during ART to expose infected cells, while creating an arsenal of immune effector cells, such as cytotoxic T lymphocytes (CTL), capable of eliminating these targets. While pharmacological latency reversal agents (LRAs) have achieved limited success in *ex vivo* studies, none have been shown to reduce the latent reservoir in HIV-1-infected individuals. In addition, some LRAs have been shown to negatively impact antigen-specific $CD8^+$ T cell effector responses *in vitro.* An optimal cure strategy must address not only induction of proviral gene expression but also clearance of reactivated cells presenting HIV-1-associated peptide epitopes by either highly functional CTL, or through incorporation of other immune-based strategies, including broadly neutralizing antibodies, T cell vaccines, or compounds modulating pro-apoptotic pathways. Conventional dendritic cells (DC) have been safely and widely used in HIV-1 clinical trials for their capacity to induce antigen-specific T cell responses, but their HIV-1 LRA potential has been underexplored. In this dissertation, I show that antigen-presenting monocyte-derived DC generated from chronic HIV-1-infected individuals on ART were shown to induce HIV-1 LR in autologous $CD4^+$ T cells in an antigen-dependent manner. The LRA activity of DC does not

appear to be a function of unidirectional communication from DC to $CD4^+$ T cells, as DC-mediated LR was enhanced by bidirectional signaling events resulting from DC:$CD4^+$ T cell cross-talk and sharply diminished by blockade of the CD40L/CD40 helper signaling pathway. Importantly, these data demonstrate the potential of this DC-based therapeutic to promote both the antigen-specific exposure and CTL killing of exposed $CD4^+$ T cells harboring replication-competent provirus. Of public health significance, strategic inclusion of virus-associated MHC class II helper antigens in DC-based HIV-1 immunotherapies could serve both as a targeted means to safely unmask virus antigen-specific $CD4^+$ T cells harboring HIV-1, and to support CTL responses that effectively target the DC-exposed latent reservoir as a functional cure strategy.

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PREFACE

Foremost, I thank the participants of the Multicenter AIDS Cohort Study for their tireless contribution to this study and to the fight for an HIV cure. Many thanks as well to Bill Buchanan for his diplomacy, dedication to the MACS, and persistence in recruiting donors.

I would like to thank Dr. Robbie Mailliard and Dr. Charles Rinaldo for the opportunity to finish my PhD in their laboratories, as well as Drs. Velpandi Ayyavoo, Jodi Craigo, Anthea Nice, Elizabeth Janci, Simon Barratt-Boyes, and Amy Hartman for their support and encouragement during that transition. I am grateful to Dr. Mailliard and to my committee for their expertise, advice, and constructive criticism, which have been instrumental in the maturation of my project and in my development as a scientist. I am proud to have played a role in such important work.

I express my utmost appreciation to Dr. Paolo Piazza, whose thought-provoking discussions, scientific and otherwise, have been invaluable to me personally and as a researcher. In addition, many thanks to Dr. Tatiana Garcia-Bates and Dr. Mariana Palma for their mentorship and for always making the time to help me solve any problem I encountered efficiently and correctly. I also thank Ming Ding and Holly Bilben for their technical assistance and flexibility.

I thank Dr. Mailliard not only for his guidance, but also for affording me the space and time to make my own path. Thank you for your calm under pressure, for standing up for me, and for making me laugh all of the times I wanted to cry.

Finally, I would like to thank my family, whose unwavering love and support throughout this journey have made it possible. Above all, I am inexpressibly grateful to my amazing mother, whose dreams for me have always exceeded those she had for herself. This is for you.

1.0 INTRODUCTION

1.1 Mechanisms contributing to HIV-1 latency

Despite major advances in human immunodeficiency virus-1 (HIV-1) treatment and prevention since the discovery of the virus in 1983 (1), the global rate of new infections remains constant at approximately 2 million per year (2, 3). Although optimal antiretroviral therapy (ART) suppresses viremia to below the limit of detection of clinical assays, HIV-1 is managed as a chronic disease due to its persistence in a long-lived population of resting memory $CD4^+$ T cells considered to be the major reservoir (4, 5). Early treatment can limit reservoir size but not prevent its establishment, which occurs within days of initial infection (6). A biphasic decay in productively infected cells follows ART initiation. In the initial rapid phase, the majority of infected cells die with a half-life ($t_{1/2}$) of <1 day due to viral cytopathic effects or lysis by HIV-specific cytotoxic T lymphocytes (CTL) (7-9). A second population that may represent recently infected cells with unintegrated HIV (10) decays within a period of approximately 14 days (9). The second slower decay phase is marked by the death of infected macrophages, dendritic cells (DC), and partially activated $CD4^+$ T cells, which have a longer turnover rate (9). Based on the decay kinetics of these compartments, it was initially predicted that 2-3 years of ART would be sufficient to eliminate all HIV-infected cells (9). However, rebound of viremia to pretreatment levels in HIV-infected individuals who discontinued therapy provided evidence for a third long-lived population of infected cells (11).

HIV latency results from infection of activated $CD4^+$ T cells that are either reverting to a resting memory state or undergoing an effector-to-memory transition (12). During this rare window of opportunity, high CCR5 expression, adequate deoxynucleotide triphosphate (dNTP) pools, and sequestration of host transcription factors permit virus entry and integration but limit post-integration proviral gene expression, thus sparing these cells from viral cytopathic effects or effector-mediated clearance (12). The existence of the latent HIV-1 reservoir was confirmed by the discovery that replication-competent virus could be induced from the resting memory $CD4^+$ T cells HIV-infected individuals on long-term ART (13-15). With a $t_{1/2}$ of 44 months, this pool of infected cells would require approximately 73 years of therapy to eradicate (16). As such, the latent reservoir of inducible, replication-competent HIV-1 in ART-suppressed individuals is considered a critical barrier to a cure (17), since the lack of viral protein expression in latently infected cells allows the reservoir to escape immune surveillance.

Numerous mechanisms that would enable viral persistence during ART have been proposed. For example, lack of expression of pro-apoptotic viral proteins or of retinoic acid-inducible gene I (RIG-I)-mediated detection of viral RNA could promote survival of latently infected cells (18, 19). In addition, compared to uninfected cells, increases in anti-apoptotic cellular proteins have been detected in latently HIV-infected cells (20-22). Specifically, increases in the B cell lymphoma-2 (Bcl-2) protein promote elevated levels of antioxidant molecules that protect latently infected cells from oxidative stress-induced apoptosis (20), and increases in the X-linked inhibitor of apoptosis (XIAP) protein counteract the protease activity of caspases 3, 7, and 9 (21, 22). Another study pointed to inhibition of pro-apoptotic cellular proteins as contributing to prolonged survival of HIV-infected Jurkat T cells (23).

It has also been shown that in HIV-infected individuals on suppressive cART, HIV-1 DNA preferentially integrates within actively transcribed host genes that regulate cell growth and division, thereby promoting viral persistence (24-26). Integration into the introns of such genes fosters latency through several mechanisms of transcriptional interference that contribute to repression of the HIV-1 promoter (27), including enhancer trapping, convergent transcription, and promoter occlusion (4, 28, 29). Enhancer trapping involves hijacking of the enhancer located within the 5' HIV-1 LTR for use by a proximal cellular gene promoter, thus preventing its activity on viral transcription (27). During convergent transcription, HIV-1 integrates in the opposite orientation of the host gene, potentially resulting in collision of elongation complexes initiating from the viral and cellular promoters and premature termination of transcription (4, 28). Finally, promoter occlusion occurs when RNA polymerase from an upstream cellular promoter displaces transcription factors from a downstream HIV-1 promoter of the same transcriptional orientation, preventing assembly of the viral preinitiation complex (4, 30).

In addition to the effects of viral integration site on proviral transcription, epigenetic silencing of HIV-1 gene expression also plays a significant role in the establishment and maintenance of latency through reversible modifications in chromatin structure (27). Chromatin-modifying enzymes regulate the four main mechanisms controlling DNA accessibility to transcription factors in the vicinity of the HIV-1 promoter, which is flanked by nucleosomes expressing repressive epigenetic marks in latently infected cells (29). Histone deacetylases selectively deacetylate lysine residues in the histone tails of HIV-1 promoter heterochromatin, producing a closed chromatin state inaccessible to transcription factors, whereas histone acetyltransferases acetylate these residues, creating an open chromatin environment conducive to proviral transcription (27). Trimethylation of histones in the HIV-1 LTR region by histone

methyltransferases also represses transcription (27), and methylation of CpG islands surrounding the HIV-1 transcription start site by DNA methyltransferases has been shown to completely silence the HIV-1 promoter (31, 32). Indeed, the correlation between cART duration and degree of methylation of the HIV-1 promoter suggests a role for this mechanism in persistence of the latent reservoir (33). Another factor contributing to repression of HIV-1 transcription during latent infection is sequestration of key cellular transcription factors, such as NF-κB, Sp-1, AP-1, and P-TEFb; that remain in an inactive state in resting $CD4^+$ T cells harboring the virus (27, 34, 35).

To further complicate the quest for a cure, latently infected $CD4^+$ T cells that may contain intact, replication-competent provirus can undergo clonal expansion due to homeostatic proliferation even during ART, thus slowing viral decay (25, 36-38). HIV-1 integration occurs at numerous sites in the host genome, favoring actively transcribed genes that become activated upon HIV infection (26, 39, 40). Analysis of integration site sequences is used to identify clonally expanded progeny arising from a single infected cell (25). Since proviral/host junction sequences identify the location and orientation of HIV DNA integration, breakpoints in host DNA can be used to identify these integration sites in clonally expanded populations (25). Longitudinal analyses of patient sequences have revealed that the proportion of persistent proviruses in proliferating cells increases with ART duration (37, 41) and that expanded clones can persist for several years (25). Furthermore, integrations are concentrated in genes associated with cell growth, mitosis, and cancer (25, 37). One study of five ART-treated individuals determined that 40% of HIV integration sites were in clonally expanded cells, and 50% of the infected cells in one patient were derived from a single clone (25). Some have reported that the majority of proliferating clonally expanded cells contain defective proviruses, while the replication-competent HIV-1 reservoir is contained within a subset of $CD4^+$ T cells characterized by single integration events

within transcriptionally silent regions of the genome (41). However, Simonetti *et al.* identified a $CD4^+$ T cell clone that contained infectious provirus and was highly enriched in cancer metastases in an HIV-infected individual with squamous cell carcinoma (36). Recent *ex vivo* data showed that T cell clones harboring replication-competent HIV-1 either persist over a span of years in some virally suppressed individuals or emerge and wane (42).

Although extensive evidence supports the lack of sequence evolution in the latent HIV-1 reservoir due to successful ART (43-47), several controversial explanations for the stability of the latent reservoir in ART-treated patients have been proposed. One report attributes continual replenishment of the reservoir during ART to ongoing, low level viral replication (48). Potential variables permitting *ne novo* infection events include suboptimal drug penetration in tissue sites such as the lymph nodes (49, 50) and cell-to-cell spread of virus that is not easily blocked by ART (51). Lorenzo-Redondo *et al.* reported ongoing HIV-1 replication in the lymphoid tissues of three cART-suppressed individuals, a phenomenon that has yet to be confirmed by other studies (27, 50). Importantly, this study purports that viral evolution is ongoing during suppressive ART based only on sequences analyzed during the first 6 months of ART, and the rapidly decaying population of infected cells that predominates during this period does not reflect the stable reservoir during chronic HIV infection (10, 52). However, another group described proviruses with genetic and transcriptional signatures of active replication in lymph node T follicular helper (T_{FH}) and non-T_{FH} subsets in elite controllers (53). In support of these findings, persistent viral replication has also been reported in the B cell follicles of SIV-infected rhesus macaque elite controllers (54), and $CXCR5^+PD\text{-}1^+$ T_{FH} have been described as the major source of replication-competent, infectious HIV-1 among blood and lymph node memory $CD4^+$ T cell populations in individuals on long-term

ART (55). In short, the extent to which viral replication in anatomical sanctuaries poses a barrier to an HIV cure warrants further investigation.

1.2 Methods to quantify the latent HIV-1 reservoir

Only replication-competent proviruses capable of viral rebound in the absence of antiretroviral therapy pose a barrier to an HIV-1 cure (5, 14). This subset of proviruses is defined as the latent HIV reservoir. However, a complex and heterogeneous population of both replication-competent and defective proviruses exists within resting $CD4^+$ T cells that harbor persistent virus (41, 56-63). Because persistent HIV-1 includes all forms of integrated proviral DNA in infected cells (64), methods of detection that cannot distinguish between these various forms prevent accurate quantification of the latent reservoir. There is little correlation between PCR- and cell culture-based assays commonly used to measure the latent reservoir, which complicates efforts to determine the efficacy of HIV eradication strategies (17).

1.2.1 Culture-based assays

The first method used to quantify the latent reservoir was the quantitative viral outgrowth assay (QVOA) (14, 65). The QVOA is a limiting dilution assay that measures the frequency of resting $CD4^+$ T cells that produce infectious virus after a single round of *in vitro* mitogenic stimulation with phytohemagglutinin (PHA) to induce maximum T cell activation. Irradiated allogeneic PBMC are added to the culture to enhance PHA-induced T cell activation by acting as antigen-presenting cells, and viruses released into the supernatant are expanded in culture for 2-3 weeks

by the addition of $CD4^+$ lymphoblasts from HIV-negative donors (14, 65). Culture supernatants are tested by ELISA for the presence of HIV-1 p24 antigen to detect exponential viral outgrowth (17, 57). Virus in the supernatant can also be detected by ultrasensitive RT-PCR at day 7 post-stimulation (66). Limiting dilution analysis software employing maximum likelihood estimates (67) is used to calculate infectious units per million (IUPM) cells recovered from the QVOA. The QVOA detects only replication-competent HIV-1 induced after one round of mitogenic stimulation (induced proviruses), but underestimates the true size of the reservoir (17). For example, viral outgrowth may not be detected in some wells that actually contain cells harboring intact provirus that might be activated after additional rounds of stimulation (noninduced proviruses), even though most noninduced proviruses are defective (57). Indeed, <1% of replication-competent proviruses are induced by maximum T cell activation (57). Thus, the QVOA estimates the frequency of replication-competent proviruses in ART-suppressed HIV-infected individuals to be 1 in 10^6 resting $CD4^+$ T cells, which is considered to be a 'definitive minimal estimate' (17, 56). Interestingly, sequencing analyses of intact viral genomes suggest that the true size of the latent reservoir is more likely $60/10^6$ resting $CD4^+$ T cells (57). Furthermore, the QVOA also has numerous technical limitations. In addition to the requirement for large blood volumes to isolate donor resting $CD4^+$ T cells, the QVOA is labor intensive, time consuming, and expensive. Although these factors have prompted the development of simpler PCR-based methods of detection, the QVOA is still considered the gold standard for quantifying the latent reservoir. Importantly, the QVOA can detect individual latently infected cells, since clonal viruses that expand in wells cultured at limiting dilution can be sequenced (17).

Variations of the QVOA have been devised, including the multiple stimulation viral growth assay (MS-VOA) (63), the latency clearance assay (LCA) (68, 69), and the murine viral outgrowth

assay (MVOA) (70). The MS-VOA was developed in recognition of the finding that the QVOA underestimates the size of the latent reservoir, since latently infected cells can proliferate in response to mitogenic stimulation in the absence of virus production, but generate progeny cells that contain infectious noninduced proviruses (INPs) (63). To increase the chances of detecting replication-competent proviruses that were not reactivated after one round of stimulation, three additional rounds of stimulation with PHA were added to the original assay format (63). The LCA is a standard QVOA that incorporates autologous CTL for the purpose of demonstrating a reduction in the recovery of replication-competent virus resulting from effector-mediated elimination of exposed virus-expressing target cells (68). Sung *et al.* utilized the LCA to demonstrate that the HDACi vorinostat can create sufficient viral antigen exposure in latently infected cells from aviremic individuals to allow clearance of these cells (69). Reported to be more sensitive than the standard QVOA (71), the MVOA has also been utilized for the amplification of latent infectious virus (70). In this assay, resting $CD4^+$ T cells from virally suppressed HIV-infected individuals are adoptively transferred into NOD-SCID IL-2R$\gamma^{-/-}$ (NSG) humanized mice, and plasma viral RNA is monitored to detect viral rebound (70). However, the MVOA is not a quantitative measurement of the latent reservoir, but rather a measure of residual viremia in peripheral blood (70). Most recent in the development of more sensitive assays to accurately quantify inducible, replication-competent HIV-1 is the TZA (72). The TZA utilizes the HIV-permissive TZM-bl reporter cell line to demonstrate productive infection by reactivated HIV-1 from latently infected resting $CD4^+$ T cells of aviremic individuals on ART (72). HeLa-derived TZM-bl cells express the HIV-1 receptor CD4, as well as CCR5 and CXCR4 coreceptors. An integrated copy of the β-galactosidase (β-gal) gene under control of the HIV-1 promoter allows detection of infectious virus through chemiluminescent measurement of β-gal activity. Fractional

provirus expression (fPVE) can be calculated as a function of IUPM divided by the total HIV-1 DNA present in a sample (72). Using this method, Sanyal *et al.* determined that the size of the inducible, replication-competent HIV-1 reservoir in ART-suppressed individuals is approximately 70-fold larger than estimates obtained from the QVOA (72), in agreement with estimates based on sequence analysis of intact proviruses (57).

Still other culture-based methods of measuring the latent reservoir rely on qRT-PCR-based quantification of different forms of viral RNA representing distinct stages of HIV-1 replication (17). In these assays, maximally stimulated resting $CD4^+$ T cells are plated at limiting dilution to include less than one virus-producing cell per well and cultured for 2-7 days. Cell-associated (CA) multiply spliced mRNA represents transcripts that are produced early in HIV replication, whereas CA unspliced transcripts are generated at later stages (17). Since HIV-1 mRNA in the culture supernatant represents virion release, it is considered to be a more accurate measure of the latent reservoir (17). However, T cell activation assays with viral RNA readout also involve a single round of stimulation and therefore cannot detect INPs. Furthermore, qRT-PCR measures of viral RNA in these assays may detect defective proviruses that are not capable of productive infection. Due to the potential of obtaining both false-negative and false-positive results, these assays cannot accurately quantify the latent reservoir (17).

1.2.2 PCR-based assays

Unlike the VOA, PCR-based assays will detect both induced and noninduced proviruses and therefore overestimate the true size of the latent reservoir, having shown that approximately $300/10^6$ resting $CD4^+$ T cells contain HIV-1 DNA (56). Numerous PCR assays have been designed for the detection of specific forms of proviral DNA that exist within HIV-infected individuals.

Total proviral DNA includes replication-competent and defective proviruses, as well as unintegrated forms of HIV-1 DNA (17). HIV DNA is commonly detected by qPCR using primers based on conserved regions of the HIV-1 genome (17). This method provides quantitation of proviral DNA relative to a standard curve rather than an absolute value (73-85). Droplet digital PCR (ddPCR) is a more precise assay for the absolute quantitation of total HIV-1 DNA that is based on water-oil emulsion partitioning of DNA into droplets, such that amplification of template molecules occurs in each individual droplet (56, 86-88). Compared to traditional PCR, this method permits the measurement of thousands of independent amplification events within a sample.

As a result of studies suggesting that linear HIV-1 DNA that fails to integrate into the host genome is unstable (89, 90), assays measuring the latent reservoir should detect only stably integrated proviruses. PCR assays that can distinguish integrated from extrachromosomal HIV DNA have become useful in addressing this issue. Among these, Alu-PCR has become the most common method for selectively amplifying integrated proviruses (91-95). This assay employs primers that target Alu elements, which are short sequences of transposable DNA found in high copy numbers within the human genome, and the HIV-1 *gag* gene (17). Subsequently, nested RT-PCR using primers in the HIV-1 LTR is performed (17). Alu-PCR is useful for detection of integrated proviral DNA in untreated viremic individuals, in whom most HIV-1 DNA exists in a linear, unintegrated form (96). Disadvantages of Alu-PCR include the inability to detect proviruses integrated too far from an Alu sequence to be amplified, and the ability to detect defective proviruses.

Other forms of unintegrated HIV-1 DNA that do not contribute to latency are 1- and 2-LTR circles. 1-LTR circles are formed as a result of failed integration and subsequent homologous recombination between the two LTRs of linear HIV DNA (97), whereas 2-LTR circles result from

non-homologous end joining between LTRs (98). Though controversial, 2-LTR circles are considered a measure of ongoing viral replication or recent infection, based on the assumption that they are labile (75, 99-101). Both qPCR (75, 102, 103) and ddPCR (56, 88) assays have been developed to detect 2-LTR circles in resting $CD4^+$ T cells and PBMC, but 1-LTR circles have proved difficult to quantify by PCR-based methods (104-106).

Due to the fact that ongoing viral replication occurs in HIV-infected individuals on suppressive ART (107), much effort has been devoted to the development of more sensitive assays to measure residual viremia. Whereas the limit of detection of most clinical assays is 50 copies of HIV-1 RNA/mL plasma, highly sensitive single-copy assays (SCA) capable of detecting as low as 1 copy/mL plasma (108-110) have recently been developed. The RT-PCR SCA assay uses primers based on conserved regions of HIV-1 *gag* to measure ongoing viral replication originating from $CD4^+$ T cells and other cellular reservoirs (45). A revised version of the SCA employing primers for a highly conserved region of integrase in the HIV-1 *pol* gene and larger volumes of plasma reported increased sensitivity compared to the *gag* SCA (111). However, the SCA is time-consuming and has a limited dynamic range due to the fact that HIV-1 RNA levels in most ART-suppressed individuals are near the limit of detection of the assay (17). Furthermore, since the SCA measures residual plasma viremia but excludes cell-associated proviral DNA, it is not a direct measure of the latent HIV reservoir. In summary, accurate quantification of the latent reservoir is unachievable with current culture- and PCR-based assays.

1.2.3 Single-cell characterization of latent HIV-1

Shortcomings of standard assays to quantify the latent reservoir have led to the advent of techniques that aim to detect latent HIV-1 on a single-cell level. This highly precise technology

combines fluorescence *in situ* hybridization for HIV-1 gene-specific mRNA (112) and flow cytometry staining for HIV Gag protein to allow simultaneous detection of HIV transcription and translation products in $CD4^+$ T cells harboring inducible latent provirus (113, 114). Commercially available as PrimeFlow™ (RNAflow) and RNAscope® ISH (RNAscope), these assays employ multiple oligomeric probes and branched DNA signal amplification to enhance detection sensitivity (113, 114). This approach has been used to evaluate the efficacy of LRAs in reactivating latent HIV-1 in either *in vitro* models of latency or $CD4^+$ T cells from HIV-infected individuals (113, 114). For example, Baxter *et al.* used *gag* and *pol* probe sets for HIV-1 mRNA in combination with flow cytometry staining for phenotypic markers and intracellular Gag to identify the transcription- and translation-competent reservoir in latently infected cells from HIV-infected individuals following treatment with various LRAs (113, 115). Evaluation of RNAflow and RNAscope using LRA stimulation in the J-Lat cell model showed that RNAscope is more sensitive and specific, with a lower limit of detection than RNAflow (114). Next-generation *in situ* hybridization techniques combining detection of viral RNA-positive (vRNA+) (RNAscope) and vDNA+ (DNAscope) cells and immunohistochemistry are being used to characterize tissue reservoirs of latent HIV and SIV infection (113, 116). Importantly, this powerful technology permits discrimination of infected vDNA+ cells that are actively transcribing HIV mRNA (vRNA+) from those that are transcriptionally silent (vRNA-) (117), which has been instrumental in demonstrating that ongoing viral replication occurs in anatomical sanctuaries (54, 118).

1.3 Therapeutic approaches to HIV-1 latency reversal

The 'kick and kill' (or 'shock and kill') approach to controlling HIV-1 involves inducing HIV-1 latency reversal (LR) during ART to expose infected cells, while creating an arsenal of immune effector cells, such as CTL, capable of eliminating these targets (119). Finding an effective means to expose and purge the latent reservoir in a nontoxic manner has been elusive and remains a major hurdle to this cure approach. Though pharmacological latency reversal agents (LRAs) have achieved limited success in *ex vivo* studies, in over 15 clinical trials none have been shown to reduce the latent reservoir in HIV-1-infected individuals (120-122). The first HIV eradication trials were based on the knowledge that induction of latent provirus is dependent on T cell activation (9, 89). Two human trials utilizing anti-human CD3 (OKT3) in combination with IL-2 not only failed to produce reductions in the reservoir but also proved toxic to participants, prompting discontinuation of the use of global T cell activating agents in subsequent studies (123, 124). Summarized in Table 1, current LRAs can be divided into mechanistic classes that include epigenetic modifiers, protein kinase C (PKC) agonists, NF-κB agonists, phosphoinisitide-3-kinase (PI3K)/Akt pathway inhibitors, T cell receptor (TCR) activators, toll-like receptor (TLR) agonists, and various unclassified compounds (125).

Table 1. Classes of latency reversal agents

LRA	Class	Mechanism(s) of action	References
HDACi	epigenetic modifiers	acetylation of histones	125, 127-132
HMTi	epigenetic modifiers	demethylation of histones	125, 127, 133-136
DNMTi	epigenetic modifiers	demethylation of DNA	32, 125, 127, 137
BETi	epigenetic modifiers	inhibit BRD4 interaction with active P-TEFb; P-TEFb releasing agent	125, 138-144
P-TEFb activators	epigenetic modifiers	phosphorylation of CDK9	125, 145
HMBA	epigenetic modifiers	phosphorylation of RNA Pol II; BRD4 activation; chromatin remodeling; P-TEFb releasing agent	138, 146-149
Bryostatin-1	PKC agonists	mimic DAG; activate PKC isoforms to initiate downstream NF-κB signaling	115, 125, 126, 159, 164
Prostratin	PKC agonists	mimic DAG; activate PKC isoforms to initiate downstream NF-κB/AP-1 signaling	125, 126, 150, 154, 155
Ingenols	PKC agonists	mimic DAG; activate PKC isoforms to initiate downstream NF-κB signaling	125, 126, 167-171
Smac mimetics	NF-κB agonists	inhibit cIAP1 to prevent degradation of NIK (NF-κB-inducing kinase)	125, 126, 174
Immune checkpoint blockers	TCR activators	TCR signaling	125, 190
Disulfiram	PI3K/Akt pathway	depletes PTEN to activate Akt signaling and NF-κB-mediated transcription	125, 126, 129, 178, 179, 181
Benzotriazole	PI3K/Akt pathway	STAT5 signaling	126, 182-184
Rapamycin	PI3K/Akt pathway	mTOR inhibitor; disrupts formation of mTORC1	125, 126, 185, 186
TLR7 agonists	TLR agonists	MyD88-dependent NF-κB and JNK signaling	125, 191-193
TLR9 agonists	TLR agonists	MyD88-dependent NF-κB and JNK signaling	125, 194
Quinolones	unclassified	inhibit BRD4 interaction with active P-TEFb; P-TEFb releasing agent	125, 213-215
IL-15	unclassified	unknown	125, 212
Galectin-9	unclassified	NF-κB, AP-1, NFAT signaling; inhibits expression of repressive chromatin-modifying factors	221

Akt, protein kinase B; AP-1, activator protein 1; BETi, bromodomain and extraterminal bromodomain inhibitors; BRD4, bromodomain-containing protein 4; DAG, diacylglycerol; DNMTi, DNA methyltransferase inhibitors; HDACi, histone deacetylase inhibitors; HMBA, hexamethylene bisacetamide; HMTi, histone methyltransferase inhibitors; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; MyD88, myeloid differentiation primary respnse 88; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κ light chain-enhancer of activated B cells; PKC, protein kinase C; P-TEFb, positive transcription elongation factor b; PTEN, phosphatase and tensin homolog; STAT5, signal transducer and activator of transcription 5.

1.3.1 Epigenetic modifiers

Epigenetic modifiers include histone deacetylase inhibitors (HDACi), histone methyltransferase inhibitors (HMTi), DNA methyltransferase inhibitors (DNMTi), bromodomain and extraterminal (BET) bromodomain inhibitors (BETi), positive transcription elongation factor b (P-TEFb) activators, and HMBA (hexamethylene bisacetamide) (125, 126). All of these compounds operate at various levels of transcriptional control to mediate changes in chromatin structure that permit proviral reactivation.

HDACi facilitate acetylation of histones, an activating epigenetic mark characteristic of open chromatin (127). Within this class of LRAs, HDACi have been most extensively studied, and clinical trials of vorinostat, panobinostat, and romidepsin have all demonstrated HIV reactivation *in vivo* (128-132). However, none of these agents impacted levels of HIV-1 DNA. HMTi and DNMTi interfere with methylation of histones and DNA, respectively, which are repressive epigenetic marks of closed chromatin (125, 127). Several inhibitors of histone methyltransferases (HMT) associated with proviral silencing including SUV39H1, G9a, and EZH2, have facilitated HIV-1 latency reversal in model cell lines and in resting $CD4^+$ T cells *ex vivo* (133-136). DNMTi that have been investigated in clinical trials for various forms of cancer are currently being explored for their antilatency potential. Specifically, decitabine (Aza-CdR) and its analog azacitidine (Vidaza®) have demonstrated HIV-1 reactivation in various *in vitro* models of latency (32, 137). Classified as P-TEFb releasing agents, BETi block the BET bromodomain interaction of bromodomain-containing protein 4 (BRD4) with acetylated histones that facilitates P-TEFb recruitment to the HIV-1 LTR in the absence of Tat (138), thereby promoting Tat-mediated recruitment of P-TEFb to the viral promoter (139-141). BETi also

mediate P-TEFb release from the repressive 7SK small nuclear ribonuleoprotein (snRNP) complex (139). In addition, BETi compounds including JQ1, I-BET, I-BET151, and MS417 have been shown to induce HIV LR in both *in vitro* models of latency as well as in primary cells from ART-suppressed individuals *ex vivo* (139, 140, 142-144).

LRAs that directly activate P-TEFb are also under investigation. During HIV transcription, the switch from initiation to elongation occurs upon successful recruitment of P-TEFb by Tat to the TAR element within the nascent HIV transcript, which is mediated partly by phosphorylation of negative factors and hyperphosphorylation of the C-terminal domain of RNA polymerase II (127). For example, the P-TEFb activator Amt87 facilitates this switch by phosphorylating the cyclin-dependent kinase 9 (Cdk9) subunit of P-TEFb, thereby releasing P-TEFb from the 7SK snRNP and inducing assembly of the Tat-super elongation complex (SEC) at the HIV-1 promoter (145). Finally, the P-TEFb releasing agent (138) HMBA induces Tat-independent proviral transcription through Cdk9-dependent phosphorylation of the C-terminal domain of RNA Pol II and subsequent recruitment of Cdk9 by Sp1 to the HIV-1 LTR (146, 147). HMBA has also been shown to activate the P-TEFb recruitment factor BRD4 (148, 149) and to mediate chromatin remodeling at nucleosome 1 (Nuc-1) near the HIV transcription start site (TSS), independent of histone acetylation or methylation (146).

1.3.2 PKC agonists

PKC agonists induce HIV LR through activation of NF-κB signaling by intracellular PKC isoforms (126). These compounds mimic the second messenger diacyglycerol (DAG), which activates PKC enzymes to initiate downstream signaling, leading to NF-κB binding at the LTR and viral transcription. PKC agonists can be divided into three chemical families, including phorbol esters,

macrocyclic lactones, and diterpenes (126). Phorbol esters under investigation include phorbol 12-myristate 13-acetate (PMA), prostratin, and 12-deoxyphorbol 13-phenylacetate (DPP), all of which operate through NF-κB and Ap-1 signaling pathways to reactivate latent provirus (150). Despite its effectiveness as a strong inducer of T cell activation and HIV transcription, PMA is not a candidate for clinical LR trials due to its oncogenic properties (151-153). However, both prostratin and DPP have demonstrated potential as T cell activators and LRAs in latently infected primary cells *ex vivo* (154, 155).

Originally isolated from the marine sponge *Bugula neritina*, the macrocyclic lactone bryostatin-1 has been implemented extensively in clinical oncology trials for its anti-neoplastic properties (156-158). Although the potent PKC agonist was shown to achieve T cell activation comparable to that induced by PHA/Il-2 stimulation *in vitro* and *ex vivo* (120, 159), its use in cancer clinical trials resulted in serious adverse events (160-163). Furthermore, in a phase I HIV-1 clinical trial, tolerable but conservative drug dosing of bryostatin-1 prevented it from reaching detectable systemic concentrations associated with PKC activation and from reactivating latent HIV-1 reservoirs (164).

The third family of PKC agonists, the diterpenes, includes ingenol compounds first isolated from the *Euphorbia* family of plants (126). In addition to their numerous medicinal properties (165), ingenols were discovered to inhibit HIV-1 replication by blocking viral adsorption through downregulation of CD4 and viral coreceptors CCR5 and CXCR4 (166, 167). Synthetic ingenol compounds have shown promise as LRAs in various models of *in vitro* latency (167, 168) and in nonhuman primates (169). The FDA-approved actinic keratosis drug ingenol-3 angelate (PEP0005) (170) exhibited synergism with the BETi/P-TEFb agonist JQ1 in reactivating latent HIV in primary $CD4^+$ T cells from ART-suppressed individuals (171). Furthermore,

coadministration of Ingenol B and vorinostat to rhesus macaques resulted in increased SIV viral load in both the periphery and the CNS (169). However, the combined LR activity of Ingenol B and vorinostat in this study was also accompanied by systemic and CNS inflammation. In concordance with these results, subsequent *in vitro* data suggest that the PKC agonists prostratin and bryostatin-1 may promote blood-brain barrier disruption and transmigration of proinflammatory leukocytes into the CNS (172).

1.3.3 NF-κB agonists

Among NF-κB agonists, second mitochondrial-derived activator of caspases (Smac) mimetics are a class of LRAs that act via the non-canonical NF-κB pathway to mediate proviral transcription (126). Smac is known as a mediator of apoptosome formation and activator of the caspase cascade in mitochondrial apoptosis pathway (125, 173). In HIV latency reversal, Smac mimetics inhibit the cellular inhibitor of apoptosis protein 1 (cIAP1) that ubiquitinates NF-κB-inducing kinase (NIK). NIK is required for the phosphorylation of IκB kinase alpha (IKKα), which in turn processes p100 into p52, resulting in the formation of RelB/p52 heteodimers that can translocate to the nucleus to induce NF-κB-dependent transcription (126). Thus, Smac mimetics prevent degradation of NIK, which culminates in NF-κB-dependent transcription and coincident induction of integrated provirus (174).

1.3.4 PI3K/Akt pathway inhibitors

Compounds that target the phosphoinisitide-3-kinase (PI3K)/Akt pathway are also being investigated for their potential as LRAs. *In vivo*, activation of PI3K/Akt signaling promotes cell

survival by blocking steps in the apoptosis pathway (175-177). Three mechanistically distinct classes of LRA candidates have demonstrated PI3K/Akt-dependent reactivation of latent HIV-1.

An FDA-approved drug used for the treatment of alcoholism, the acetaldehyde dehydrogenase inhibitor disulfiram was first shown to induce proviral transcription in a primary cell model of latency (178). Disulfiram reactivates NF-κB-dependent viral gene expression by depleting the phosphatase and tensin homolog (PTEN), a tumor suppressor that negatively regulates intracellular levels of phosphatidylinositol-3, 4, 5-triphosphate (PIP3) and the Akt signaling pathway (179). Based on the *in vitro* finding that disulfiram could induce LR in the absence of global T cell activation, the drug has been tested in human clinical trials, which demonstrated slight increases in viral RNA (180, 181) that were accompanied by a 14% decrease in reservoir size (181).

Benzotriazole derivatives reactivate latent HIV through a mechanism that involves γc-cytokine-dependent phosphorylation of the transcription factor STAT5 (signal transducer and activator of transcription 5) (182). Sustained phosphorylation of STAT5 is required for its activation and translocation to cognate binding sites within the viral promoter (183). Covalent addition of the protein SUMO2/3 (Small Ubiquitin-like Modifier 2/3) to STAT5 prevents phosphorylation and nuclear import of STAT5. Benzotriazoles inhibit this interaction to permit STAT5-mediated HIV transcription (182). Importantly, benzotriazoles have been reported to reactivate latent HIV-1 *in vitro* and *ex vivo* without toxicity or global T cell activation (184).

Finally, the mTOR (mammalian target of rapamycin) inhibitor rapamycin has been investigated not as an LRA itself, but for its ability to mitigate the cytokine-associated toxicity and proliferative effects of potent T cell stimulating agents employed in latency reversal strategies (185). Rapamycin is an immunosuppressive compound that exerts its effects on the PI3K/Akt

pathway downstream of IL-2 signaling (186) by disrupting formation of mTOR complex 1 (mTORC1), which is a regulator of cellular metabolism and activation. Martin *et al.* determined that rapamycin downregulated proinflammatory cytokine release and cellular proliferation in anti-CD3/CD28-activated resting $CD4^+$ T cells from ART-suppressed individuals but did not inhibit HIV-1 reactivation (185). Of relevance to 'shock and kill' approaches for targeting the latent reservoir, rapamycin did not impair CTL effector function (185).

1.3.5 TCR activators

The TCR-activating class of LRAs includes immune checkpoint (IC) inhibitors (ICIs). It has been hypothesized that ICIs used for reversal of exhaustion in HIV-specific T cells displaying ICs could potentially de-repress HIV latency in $CD4^+$ T cells coexpressing these markers (187). Two previous cases of HIV-infected cancer patients treated with either Programmed Cell Death-1 (PD-1) or Cytotoxic T Lymphocyte-associated Protein-4 (CTLA-4) monoclonal antibodies reported no effects on latent HIV reservoirs (188, 189). However, to date one HIV-infected individual treated with anti-PD-1 (nivolumab) for non-small-cell lung cancer experienced increases in plasma viral load and decreases in cell-associated HIV DNA that were accompanied by increases in $CD8^+$ T cell function and decreased $CD8^+$ T cell exhaustion, suggesting that nivolumab induced synergistic 'shock and kill' effects in this patient (190).

1.3.6 TLR agonists

The LR potential of TLR7 agonists has been investigated in several *ex vivo* and *in vivo* studies (191-193). The compound GS-9620 induced extracellular HIV-1 RNA in PBMCs of virally

suppressed individuals, increased cytolytic activity of HIV-specific $CD8^+$ T cells, and enhanced killing of infected cells mediated by the HIV envelope-specific broadly neutralizing antibody (bNAb) PGT121 (191). In these studies, both latency reversal and T cell activation were dependent on type I IFNs produced through TLR7 stimulation of pDC. Of note, GS-9620 was also able to induce polyfunctional T cell cytokine responses independent of antigen specificity in PBMC from HIV-naïve individuals in response to CEFT peptides.

More recently, TLR7 agonists have been employed *in vivo* to target the latent reservoir. Therapeutic vaccination with Ad26/MVA (recombinant adenovirus serotype 26 prime/modified vaccinia Ankara boost) expressing SIV_{smE543} *gag-pol-env* and stimulation with the TLR7 agonist GS-986 increased the breadth of SIV-specific CTL responses, decreased levels of lymph node and peripheral blood SIV DNA, and delayed viral rebound following ART interruption in SIV_{mac251}-infected rhesus macaques (192). These effects were attributed to the combination of therapeutic vaccination and innate immune stimulation and could not be achieved by TLR7 stimulation alone. In a study of SHIV-SF162P3-infected rhesus macaques, administration of the HIV-1 Env-specific bNAb PGT121 in combination with TLR7 agonist GS-9620 during ART delayed viral rebound upon ART discontinuation (193). Adoptive transfer of lymph node mononuclear cells from monkeys that did not rebound during the study period did not transfer infection to SHIV-naïve monkeys. Furthermore, viral loads remained undetectable in monkeys that did not rebound following CD8 depletion. Importantly, this study highlights the potential of a bNAb-TLR7 agonist combination to target the viral reservoir, even in the absence of therapeutic levels of bNAbs following discontinuation of ART (193). Now termed vesatolimod, GS-9620 has advanced to Phase 1b clinical trials. To be completed in February 2019, clinical trial NCT02858401 (http://www.clinical trials.gov NCT02858401) is a placebo-controlled dose-escalation study

investigating the safety and tolerability of vesatolimod in HIV-1-infected, ART-suppressed participants and its effect on changes in plasma HIV-1 RNA. Another current placebo-controlled trial of vesatolimid (http://www.clinical trials.gov NCT03060447) is investigating the safety and efficacy of a 10-dose regimen in ART-treated HIV-1-infected controllers on ART and during analytic treatment interruption (ATI).

The efficacy of TLR9 agonist MGN1703 as an innate immunity enhancer and LRA was evaluated in a single-arm, open-label study (http://www.clinical trials.gov NCT02443935) of HIV-1-infected, ART-suppressed individuals who were administered the drug subcutaneously twice weekly for 4 weeks (194). Study outcome measures included assessment of pDC, NK, and T cell activation; plasma HIV-1 RNA and cytokine levels, and interferon-stimulated gene (ISG) expression. Activation of pDC, increases in plasma IFN-2α, upregulation of ISG transcription in $CD4^+$ T cells, and increased proportions of activated NK and $CD8^+$ T cells resulted from MGN1703 treatment. Increases in plasma HIV-1 RNA > 1500 copies/mL were also detected in 6 of 15 participants. However, MGN1703 dosing did not impact reservoir size, as measured by levels of total and integrated HIV-1 DNA (194). The upcoming clinical trial TITAN (http://www.clinical trials.gov NCT03837756) will evaluate the safety and efficacy of TLR9 agonist lefitolimod in combination with the bNAb 3BNC117/10-1074 in HIV-1-infected individuals on ART and during ATI, and its impact on HIV-1 reservoir reduction.

In earlier studies, TLR5 and TLR1/2 agonists were explored as potential LRAs. The TLR5 agonist flagellin was shown to induce NF-κB-dependent transcription of HIV RNA from healthy donor resting central memory $CD4^+$ T cells (T_{CM}) that were *in vitro* infected with recombinant luciferase-encoding HIV-1 particles (195). Since T_{CM} constitute the major latent HIV-1 reservoir *in vivo*, and gut-associated lymphoid tissue (GALT) contains the largest pool of T_{CM}, this study

supports previous findings that microbial translocation and low-level proliferation of T_{CM} in the gut contribute to chronic immune activation and virus dissemination in HIV-1-infected individuals (82, 195, 196). However, flagellin could not reactivate latent provirus in resting $CD4^+$ T cells from aviremic individuals (195).

The TLR1/2 agonist Pam3CSK4 reactivated latent HIV-1 in an *in vitro* latency model incorporating healthy donor naïve $CD4^+$ T cells that were infected with defective virus and cultured to induce a T_{CM} phenotype (197, 198), and in T_{CM} of virally suppressed HIV-infected individuals (199). Interestingly, virus transcription was mediated by cooperative NF-κB, NFAT, and AP-1 signaling, in the absence of T cell activation and proliferation. Since Pam3CSK4 induced latent provirus selectively, it could be an attractive therapeutic target in latency reversal strategies that aim to prevent global T cell activation (199).

1.3.7 Unclassified LRAs

LRAs with unclassified mechanisms of action that are under study include the cytokine IL-15, quinoline compounds, and galectin-9 (125). IL-15 is a cytokine produced by activated dendritic cells and macrophages that activates NK and $CD8^+$ T cells to kill virus-infected cells (200-202). IL-15 is presented in *trans* to NK and $CD8^+$ T cells as part of a membrane-bound IL-15:IL-15Rα complex (201, 203), but can also be secreted as a monomer. IL-15/IL-15Rα heterodimers cleaved from DC and macrophage membranes have greater biological activity and a longer serum half-life than IL-15 monomers (204, 205), and thus complexes of recombinant IL-15 and soluble recombinant IL-15Rα have been synthesized to create IL-15 ‘superagonists’ that recapitulate the *in vivo* heterodimer functionality (206). Il-15 superagonists have been shown to promote *in vivo* NK and $CD8^+$ T cell effector responses to cancer (207-210) and to HIV-1 infection in humanized

mice (211). In a recent study, both IL-15 and the IL-15 superagonist ALT-803 induced LR activity in a primary $CD4^+$ T cell model of HIV latency (212), and ALT-803 also enhanced CTL killing of HIV-infected cells *ex vivo* (212). ALT-803 is currently in several human clinical cancer trials for solid and hematological malignancies (NCT01946789, NCT01885897, NCT02099539), and dose escalation studies are being performed to assess whether ALT-803 can be tolerated at doses determined to be safe in cynomologous macaques (212).

Quinolines were first identified as potential LRAs for their ability to reactivate latent HIV in Bcl-2-transduced primary $CD4^+$ T cells in the absence of undesirable nonspecific T cell activation (213). The quinolone compound MMQO (8-methoxy-methylquinolin) was discovered by small molecule library screening for agents capable of reacting HIV-1 in the latently infected J-Lat cell line (214). MMQO activates transcription of latent provirus by inhibiting the BRD4 protein that competes with Tat for association with the P-TEFb kinase complex (215). Thus, inhibition of BRD4 permits Tat-mediated recruitment of P-TEFb to the HIV mRNA TAR element and transcription elongation (127). In this regard, MMQO may also be classified as a novel BET inhibitor whose mechanism of action is the same as that of JQ1. Utilizing barcoded HIV ensembles (B-HIVE) technology, this study also determined that MMQO targets a different subset of HIV-1 integration sites than those targeted by HDACi or PKC agonists (215).

The human carbohydrate-binding protein galectin-9 (Gal-9) gained attention for its possible role in HIV latency reversal as a result of data demonstrating that it regulates expression of the host restriction factor and cell cycle regulator (132, 216) p21 (217-219). In addition, a recent study found that viral transcription in ART-treated HIV-infected individuals is regulated by p21 (220). Abdel-Mohsen *et al.* demonstrated that recombinant Gal-9 (rGal-9) potently reactivated latent provirus *in vitro* in J-Lat cells and *ex vivo* in $CD4^+$ T cells from virally suppressed HIV-

infected individuals (221). The mechanism of rGal-9-mediated LR involved signaling through N-linked oligosaccharides and O-linked hexasaccharides on the T cell surface, which also induced expression of the proviral transcription initiation factors NF-κB, AP-1, and NFAT, and the host antiviral restriction factor APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic subunit 3G) (221). Conversely, rGal-induced signaling inhibited gene expression of several repressive chromatin-modifying factors, including HDAC-1, -2, and -3; EZH2, SUV39H1, DNMT1, BAF, and BCL11B (221). Interestingly, plasma levels of soluble Gal-9 in HIV-infected individuals on ART correlated with cell-associated HIV RNA and with levels and binding avidity of circulating HIV-specific antibodies, which may suggest that Gal-9 mediates LR *in vivo* (221).

1.3.8 LRA combination therapy

Both *ex vivo* studies and clinical trials have demonstrated that no single pharmacological LRA in current use has been able to reactivate a significant proportion of the latent HIV-1 reservoir (152, 222). In addition, it is unclear whether multiple doses of a single LRA improve the efficacy of latency reversal, as *in vivo* studies of single and multiple dose administration of the HDACi vorinostat reported greater increases in cell-associated HIV RNA after a single administration to participants (128, 223). In an effort to address these issues, cure strategies are employing combinations of LRAs from different mechanistic classes, with the hypothesis that pharmacological synergy could facilitate increased viral reactivation with a single dose, while mitigating potential deleterious side effects of multiple dose regimens (126).

An extensive study by Laird *et al.* investigating the efficacy of LRA combinations in patient samples *ex vivo* determined that PKC agonists in combination with either HDACi or the BETi JQ1 were most effective at inducing HIV transcription compared to maximum reactivation

by PMA/ionomycin (152). In agreement with these findings, the PKC agonist/BETi LRA combinations bryostatin-1 + JQ1 and Ingenol-B + JQ1 synergized to achieve levels of *ex vivo* viral reactivation comparable to positive control stimulation using anti-CD3/CD28 antibodies in another recent study (138). Furthermore, several HMTi in combination with either JQ1 (224), the PKC agonist prostratin, or HDACi such as vorinostat (225), have enhanced proviral reactivation in both T cell lines and in resting $CD4^+$ T cells *ex vivo* (224, 225). Among atypical LRAs, galectin-9 potently reversed latency in patient cells *ex vivo* when combined with JQ1 (221). These and numerous other studies demonstrate that synergy between various LRA mechanistic classes is achievable, albeit not easily interpreted in different *in vitro* and *ex vivo* systems. It is also important to note that *in vitro* latency models routinely used to interrogate LRA function do not accurately recapitulate LR *in vivo,* and results of these studies must be interpreted with caution (132, 226, 227). As such, *in vitro* functional studies of LRA activity should be validated using relevant animal models and clinical studies to be considered physiologically relevant. Furthermore, some pharmacological LRAs, specifically histone deacetylase inhibitors and PKC modulators, inhibit the killing of HIV-1-infected cells by negatively impacting antigen-specific $CD8^+$ T cell effector responses (discussed below) (159, 226), which is an important consideration in the design of both single and combination LRA approaches. All of these findings necessitate an effective eradication strategy capable of reversing latency without global T cell activation (228) or impairment of cytolytic effector function.

1.4 Alternative approaches to the 'kick and kill' of latent HIV-1

Due to the major hurdles encountered thus far in the 'kick and kill' approach to HIV-1 eradication, numerous alternative strategies for combating latent HIV infection are gaining attention. For example, compounds shown to induce apoptosis in cancer chemotherapy are currently being explored for their potential to eliminate latently HIV-infected cells, either alone or in combination with LRAs (125). Of interest, the Bcl-2 (B cell lymphoma-2) protein family includes pro- and anti-apoptotic molecules and sensors of apoptotic stimuli that regulate apoptosis through the mitochondrial, or intrinsic, pathway (125, 229, 230). Cellular stress, DNA damage, and radiation are among triggers of apoptosis via this route, leading to mitochondrial permeabilization, apoptosome formation, and activation of the caspase cascade (173, 231). Apoptosis can also be negatively regulated by inhibitor of apoptosis (IAP) family members that prevent caspase activation (229). Bcl-2 antagonists have been investigated for use in cancer immunotherapies due to their ability to block the anti-apoptotic function of Bcl-2 family proteins, which are overexpressed in various cancers (232-234). Levels of anti-apoptotic Bcl-2 are also elevated in resting $CD4^+$ T cells upon reactivation of HIV latency (233). Bcl-2 antagonists permit HIV protease-dependent apoptosis in reactivated, latently infected resting $CD4^+$ T cells by preventing Bcl-2-mediated sequestration of the HIV protease-derived, pro-apoptotic Casp8p41 protein (233). Licensed for the treatment of chronic lymphocytic leukemia, the compound Venetoclax has been shown to reduce the frequency of latently HIV-infected, anti-CD3/CD28-reactivated T cells from ART-suppressed individuals *ex vivo* (233, 235). In addition, the potential utility of Venetoclax in reducing HIV-1 reservoir establishment was demonstrated *in vitro* through selective elimination of infected primary T cells during productive infection (236). However, the efficacy of Bcl-2

inhibitors in combination with LRAs that do not induce maximum T cell activation is unknown (125).

The PI3K pathway promotes cell survival through inhibitory phosphorylation of pro-apoptotic molecules or transcription factors by the serine/threonine kinase second messenger Akt (175-177). Since expression of HIV Nef and Tat proteins early in the virus life cycle activates PI3K/Akt signaling to prevent premature apoptosis of infected cells, inhibition of key effectors in the PI3K pathway is being explored as a potential strategy for promoting apoptosis-induced elimination of these cells (237-239). The Akt inhibitors Edelfosine, Perifosine, and Miltefosine were shown to inhibit HIV-induced Akt activation *in vitro*, resulting in death of infected macrophages (239), which constitute a long-lived viral reservoir *in vivo*. The use of the PI3K inhibitor CUDC-907 in combination with LRAs has been proposed as a 'kick and kill' strategy for its dual abilities to inhibit class I PI3K isoforms and HDAC class I and II enzymes (125, 240, 241). However, the selection of LRAs in such combination approaches warrants careful consideration, as some have demonstrated opposing effects on PI3K inhibitor activity (125). For example, the LRA disulfiram depletes a negative regulator (PTEN) of the PI3K/Akt pathway, promoting NF-κB-mediated proviral transcription and survival of infected cells (178, 242). Furthermore, the LRA activities of HDAC inhibitor vorinostat and PI3Kα isoform agonist 55704 also activate PI3K/Akt signaling (243). In fact, clinical trials of vorinostat (128) and disulfiram (180, 244) revealed no reduction in HIV DNA in virally suppressed participants. Conversely, use of PI3K inhibitors in combined strategies could negatively impact latency reversal through inhibition of transcription factors necessary for HIV-1 replication (125).

Another potential target of HIV-1 'kill' interventions is the second mitochondrial-derived activator of caspases (Smac) that mediates apoptosome formation and subsequent activation of the

caspase cascade when released into the cytosol (173, 231). Smac mimetics promote apoptosis by competitively binding and inactivating inhibitory apoptosis proteins (IAPs), such as the X-linked inhibitor of apoptosis (XIAP) that inhibits caspases 3, 7, and 9 (125). The Smac mimetic Birinipant and the XIAP antagonists GDC-0152 and Embelin produced dose-dependent increases in selective apoptosis of HIV-infected primary resting $CD4^+$ T cells without the addition of LRAs (245). Importantly, this study implies that selective elimination of latently infected cells may be achieved in the absence of reactivation. In another study, pretreatment with the Smac mimetic AEG40730 sensitized monocyte-derived macrophages to Vpr-induced apoptosis (246).

Finally, inducers of the viral RNA-sensing pattern recognition receptor (PRR) RIG-I (retinoic acid-inducible gene I) are another focus of apoptosis-mediated HIV-1 cure approaches. Detection of HIV RNA by RIG-I induces antiviral immune responses and apoptosis of infected cells (247). As retinoic acid (RA) can induce RIG-I expression and HIV transcription through activation of the transcription factor p300 acetyltransferase (19), treatment with RA mimetics could simultaneously reactivate latent provirus and promote RIG-I-mediated apoptosis of infected cells. *In vitro*, the RA derivative Acitretin was shown to induce HIV transcription and selective apoptosis of latently infected $CD4^+$ T cells of ART-suppressed individuals, as well as decreases in HIV DNA (19). In combination with Acitretin, vorinostat enhanced selective elimination of HIV-infected cells compared to uninfected cells in latently infected T cell lines (19). However, these findings could not be reproduced in a subsequent study by Garcia-Vidal *et al.* (248). Nevertheless, an attractive feature of LRA/apoptosis-inducing combination strategies is lack of dependence on effective CTL responses or complications arising from immune escape (125).

Besides pro-apoptotic compounds, the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system that functions as an adaptive immune system in bacteria and archaea

by detecting and eliminating foreign nucleic acids of invading pathogens (249-251) has also been harnessed for use in strategies to target the latent HIV-1 reservoir (252). Employing the Cas9 endonuclease of *Streptococcus pyogenes*, the modified CRISPR-Cas9 system cleaves double-stranded DNA (dsDNA) in a sequence-specific manner, mediated by a guide RNA that directs Cas9 to a complementary sequence in the target DNA (252). Compared to the nonspecific, global TCR-activating effects of most pharmacological LRAs, this approach is highly desirable. However, although this tool was widely explored in earlier genome editing HIV cure strategies to either excise proviral DNA from latently infected cells (253-255) or to render host cells less susceptible to HIV infection through knockout or knockdown of viral coreceptor expression (256-260), possible introduction of deleterious mutations resulting from error-prone NHEJ DNA repair prompted development of therapies with fewer off-target effects (252, 261). Furthermore, mutations preventing gRNA binding to target DNA sequences and use of gRNAs targeting poorly conserved proviral sequences can lead to the development of Cas9/gRNA-resistant escape variants (252). As a result, further modification of the CRISPR-Cas9 system through mutation of the Cas9 endonuclease catalytic domains has been explored for use in 'shock and kill' strategies to activate transcription of integrated HIV-1 (252, 261). The resulting 'deactivated' or 'death' Cas9 (dCas9) variant lacks dsDNA cleaving activity while retaining gRNA-mediated sequence-specific DNA binding affinity (252). Fusion of the dCas9 protein to a viral or cellular transcription factor-derived activation domain (AD) facilitates recruitment of transcription-activating and chromatin remodeling factors upon gRNA-directed targeting of the dCas9-AD fusion protein to specific promoter or enhancer sequences within the HIV-1 LTR (252). While this strategy circumvents host cell DNA damage, as with the original CRISPR-Cas9 construct, concerns remain regarding

potential off-target genetic effects and the ability to deliver the technology efficiently to all latently infected or HIV-susceptible cells *in vivo* (252).

1.5 Permanent suppression of HIV-1 reactivation as a functional cure strategy

Due to evidence that some level of ongoing viral replication occurs in the lymphoid tissues of ART-suppressed individuals (50), even more recently the focus has shifted from 'kick and kill' strategies to target the latent HIV-1 reservoir to strategies aimed at permanently repressing viral reactivation. Termed 'block and lock' (262), this cure approach targets viral proteins that are strong regulators of HIV-1 transcription. A particularly attractive candidate is the viral transactivator of transcription (Tat) protein and its interaction with the HIV RNA transactivation reponse element (TAR), whose inhibition has been the goal of several *in vitro* and *ex vivo* studies (262-267). During HIV-1 transcription, the switch from initiation to elongation occurs upon successful recruitment of positive transcription elongation factor b (P-TEFb) by Tat to the TAR element within nascent transcripts at the HIV-1 promoter (127). Thus, antagonists of Tat itself or of the Tat/TAR interaction have been explored as inducers of permanent HIV-1 latency that is refractory to viral reactivation by standard LRAs (27). Among compounds being investigated as Tat inhibitors, didehydro-Cortstatin A (dCA), an analog of the natural steroid Cortistatin A, prevents transactivation of the HIV-1 promoter by competitively binding to the TAR-binding domain of Tat (265, 266). Both *in vitro* and *ex vivo*, dCA treatment was shown to induce an almost permanent state of proviral latency that was resistant to disruption upon ART discontinuation or by potent stimulation with the PKC agonist prostratin (265, 266). Kessing *et al.* further demonstrated that dCA-mediated epigenetic silencing of the HIV-1 promoter resulted from

increased nucleosome occupancy at Nucleosome-1, and that dCA could not only suppress viral rebound after treatment interruption but also reduce tissue HIV-1 RNA levels in ART-suppressed bone marrow-liver-thymus (BLT) mice (262). Triptolide, another Tat inhibitor that has been historically used to treat rheumatoid arthritis, is currently the subject of a Phase III clinical trial (NCT02219672) investigating its impact on reservoir seeding during acute HIV-1 infection (27). Triptolide was shown to inhibit HIV-1 transcription by promoting proteasomal degradation of Tat (268). One distinct advantage of Tat-focused 'block and lock' strategies is that Tat has no cellular homologs (27), and therefore concerns regarding nonspecific or off-target effects that could result from 'shock and kill' strategies implementing pharmacological LRAs or the CRISPR-dCas9 system, respectively (261), could be circumvented.

Among potential targets of non-Tat-based latency strengthening therapies is heat shock protein 90 (HSP90), which is required for NF-κB-dependent HIV-1 reactivation under hyperthermic conditions (27, 269). Inhibitors of HSP90 were shown to block HIV-1 reactivation in J-Lat cells, supporting the role of HSP90 in latency reversal (269). Furthermore, the telomerase-derived peptide vaccine GV1001, currently in Phase II clinical cancer trials, suppressed HIV-1 reactivation by PMA *in vitro*, but its antiviral activity was completely abrogated by HSP90-neutralizing antibody (270). Another recent therapeutic strategy exploits the interaction between HIV-1 integrase (IN) and the host cofactor lens epithelium-derived growth factor (LEDGF)/p75, to retarget viral integration to transcriptionally silent regions of the genome, rendering the reservoir refractory to reactivation by various LRAs (271). This class of integration inhibitors, termed LEDGINs, bind the LEDGF/p75 binding pocket of IN (272, 273), thereby depleting LEDGF/p75 and redirecting lentiviral integration out of transcription units (274). Vranckx *et al.* demonstrated that LEDGIN treatment decreased the reactivation potential of latent provirus by LRAs including

TNF-α, suberoylanilide hydroxamic acid (SAHA), prostratin, and phorbol 12-myristate 13-acetate (PMA), in various cell lines and in primary $CD4^+$ T cells (271).

Inhibition of the HIV-1 Rev protein has also been explored as a mechanism to establish deep latency for its role in regulating the export of unspliced and singly spliced viral mRNAs from the host cell nucleus (126). The Rev inhibitor ABX464 was shown to suppress viral replication *in vitro* and in HIV-1-infected humanized mice, leading to extended control of viral rebound in ABX464-treated mice after removal of ART (275). Finally, in addition to its potential as an LRA, the aforementioned CRISPR-dCas9 system could also be modified for use as a latency strengthening agent (LSA) (261). Rather than reactivating HIV-1 gene expression in a sequence-specific manner using dCas9 activators, gRNA-directed binding of the dCas9 protein to transcription factor binding sites within the HIV LTR can be used to inhibit proviral transcription (252). Binding of the dCas9/gRNA complex to NF-κB and Sp1 sites was shown to prevent binding of these transcription factors and reduced subsequent LTR-driven gene expression (276, 277). In addition, dCas9 repressors can be designed by fusion of dCas9 to repressor domain proteins to facilitate more active repression of transcription than would be accomplished by interference with transcription factor binding alone (252). Among natural and artificially engineered repressor domains, the Kruppel-associated box (KRAB)-containing zinc finger proteins were shown to inhibit HIV LTR activity in numerous studies through targeting of the TAR element (278-284). Importantly, when combined with ART, 'latency strengthening' (261) functional cure approaches would limit chronic immune activation and inflammation associated with residual viremia (102, 285), as well as reservoir expansion facilitated by chronic inflammation (285).

1.6 Challenges for the kill phase of HIV-1 cure

Numerous clinical trials of pharmacological LRAs including vorinostat, disulfiram, bryoststin-1, panobinostat, and romidepsin, have shown that although proviral reactivation is achievable *in vivo*, elimination of reactivated cells is minimal to nonexistent (128-130, 132, 164, 180, 286). These results highlight problems inherent with the use of current LRAs in 'shock and kill' strategies to target the latent reservoir by demonstrating that the 'kill' does not necessarily follow latency reversal, regardless of sufficient expression of viral RNA or antigens by infected cells (122, 287). A successful anti-HIV memory response requires CTL recognition of cells expressing peptide antigen derived from replication-competent proviruses following latency reversal (288). HIV-1 epitopes presented by infected cells in the context of MHC class I are recognized by HIV-specific resting memory CTL that have become activated upon recall of HIV-1 antigen. Activated CTL subsequently kill infected target cells through release of perforin and granzymes. However, the challenges associated with effective killing of latently infected cells are multifactorial.

First, CTL recognition of HIV-1 epitopes is hampered by escape mutations that are selected early in infection due to heightened immune pressure (289-292) and continue to contribute to the latent reservoir until the initiation of ART (288). Proviruses with escape mutations to immunodominant CTL epitopes comprise a significant portion of the latent reservoir in individuals who initiate ART in the chronic stage of HIV-1 infection (293). Thus, successful CTL-based HIV eradication strategies must target subdominant viral epitopes for which escape mutations have not accumulated. Alternatively, selective induction of new HIV-specific CTL through priming of naïve $CD8^+$ T cells can achieve recognition of persistent HIV in individuals on ART, without targeting existing, dysfunctional memory T cells (294).

A second challenge of the 'kill' phase is the excess of defective proviruses that occupy the latent reservoir *in vivo*. These defective proviruses (HIV-1_{def}) can result from hypermutations, large internal deletions, packaging signal deletions, major splice donor (MSD) mutations, or inactivating point mutations (57, 59, 295) and significantly outnumber cells harboring replication-competent proviruses. As a result of intact LTR promoter function, integration into actively transcribed genes, and lack of promoter methylation, some HIV-1_{def} can be transcribed, translated, and recognized by HIV-specific CTL (57, 58, 295). Specifically, cells containing HIV-1_{def} with hypermutations or packaging signal/MSD mutations are decoy targets for CTLs and act to divert CTL-mediated elimination of the true latent reservoir (295). Pollack *et al.* demonstrated the phenomenon of 'cold target' inhibition using $CD4^+$ T cells transfected with reconstructed defective proviruses derived from HIV-infected individuals on ART, cocultured with autologous CTL clones specific for Gag and Nef epitopes (295). In another recent study, autologous CTL clones eliminated HIV-infected cells that had undergone IL-15-mediated latency reversal, demonstrating decreases in total HIV-1 DNA but not in inducible, replication-competent proviruses (296). It has been postulated that preferential targeting of HIV-1_{def} may be a function of their ability to present antigen to CTLs more efficiently as a result of a defect in Nef and the inability to downregulate MHC class I (295). However, the role of HIV-1_{def} *in vivo*, where the effector-to-target ratio will likely differ depending on anatomical site and not reflect the defined ratio used in *ex vivo* culture, warrants investigation (295). Of note, a study in SIV-infected macaques receiving therapeutic vaccination revealed a > 2 log decrease in HIV DNA that was accompanied by a 1 log decrease in intact proviruses, pointing to elimination of cells harboring defective proviruses (192). Evidence that clonal expansion of cells containing both defective and replication-competent HIV-1 shapes

the pool of latently infected cells in individuals on long-term ART (25, 37, 38, 41, 63) poses an additional challenge to targeting only those proviruses that are a barrier to an HIV cure.

Additional complications of 'shock and kill' approaches that utilize pharmacological LRAs for the 'shock' are the negative impact of some of these compounds on CTL effector function. Jones *et al.* demonstrated that *in vitro* exposure of $CD8^+$ T cells to the HDAC inhibitors (HDACi) vorinostat (SAHA), romidepsin, and panobinostat impaired IFN-γ production, and that even transient exposure to romidepsin or panobinostat induced selective death of activated T cells (226). HDACi impaired proliferation of HIV-specific $CD8^+$ T cells, as well as elimination of HIV-infected or peptide-pulsed target cells (226). Romidepsin exhibited the greatest inhibitory activity at all doses tested (226). These findings were subsequently confirmed by Clutton *et al.*, who determined that in comparison to HDACi, PKC modulators induced stronger T cell activation, proliferation, and nonspecific cytokine production (159). With the exception of vorinostat, all LRAs tested reduced antigen-specific $CD8^+$ T cell functions, the extent and timing of which varied between LRAs (159). LRA toxicities were a factor of both duration of exposure to drug and culture period, with bryosastin-1 being the most toxic (159). Though effective as LRAs in combination, bryostatin-1 and romidepsin (152) were shown to inhibit elimination of reactivated autologous $CD4^+$ T cells by primary HIV-1-specific $CD8^+$ T cells in both chronic progressors and elite controllers (297). In this study, bryostatin-1 or bryostatin-1/romidepsin combination treatment caused increases in $CD8^+$ T cell death, and bryostatin-1 treatment alone induced increases in expression of exhaustion markers PD-1, TIM-3, 2B4, and CD160 (297). Walker-Sperling and colleagues also confirmed downregulation of CD3 expression on $CD8^+$ T cells subject to bryostatin-1, prostratin, or bryostatin-1/romidepsin combination treatment, which could interfere with the ability of CTLs to recognize cognate antigen (297). Finally, the immunosuppressive

effects of LRAs extend beyond CTL function, as HDACi have been shown to reduce degranulation and killing capacity of NK cells (298).

CTL exhaustion is another critical consideration in the design of effective kill phase strategies. T cell exhaustion can occur in the settings of chronic viral infections or cancer, as a result of persistent immune activation and antigen exposure (288, 299-302). Among defects in CTL function that arise during chronic HIV-1 infection, exhaustion cannot be fully reversed with ART (303). Exhaustion in antigen-specific T cells involves a gradual loss of effector function, accompanied by decreased proliferative capacity (304). Compared to terminally differentiated or memory $CD8^+$ T cells, exhausted $CD8^+$ T cells are characterized by decreased cytokine production and cell surface expression of inhibitory receptors including PD-1, TIM-3, CTLA4, CD160, LAG-3, and TIGIT (304-309). Increases in inhibitory receptor expression serve as immune checkpoints (ICs) to limit excess T cell activation during a normal immune response and are downregulated upon its resolution (307, 310). Conversely, sustained expression of inhibitory receptors results from persistent antigen exposure, leading to an exhausted phenotype. Even in the context of suppressive ART, in which exposure to HIV antigen is limited, it may not be possible to completely restore CTL functionality (288). This is illustrated by high levels of PD-1 and TIGIT expression by both follicular and nonfollicular $CD8^+$ T cells in the lymph nodes of HIV-infected individuals (311). However, immune checkpoint blockade combination therapies approved for use in cancer immunotherapy have been shown to reverse exhaustion to varying degrees in virus-specific T cells (301, 309, 312, 313). In a recent study, *in vitro* blockade of the PD-1/PD-L1 axis in follicular and nonfollicular lymph node cells restored HIV-1-specific $CD8^+$ T cell function (311). Importantly, correlations between HIV DNA content in memory $CD4^+$ T cells and expression of PD-1, TIGIT, and LAG-3 inhibitory receptors have been documented, suggesting a potential role for these ICs

in latency establishment (288, 314, 315). Information gleaned from these studies could be used to boost CTL function of lymph node CXCR5-expressing $CD8^+$ T cells and subsequent targeting of the latent reservoir within this compartment (288). A phase I clinical trial of the anti-PD-L1 antibody BMS-936559 has produced encouraging results, demonstrating a trend toward enhanced Gag-specific $CD8^+$ T cell responses in virally suppressed HIV-1-infected participants (316).

Finally, one of the greatest challenges of targeting the HIV reservoir for CTL-mediated elimination is the spatial separation of target and effector cells in anatomical 'sanctuaries' (54) that serve as a persistent source of latent provirus. For example, the $CD4^+$ T follicular helper (T_{FH}) and T follicular regulatory (T_{FR}) cell subsets within B cell follicles of lymph nodes are more permissive to HIV-1 infection than extrafollicular (EF) subsets *ex vivo* (317-319) and comprise a major portion of the latent reservoir during both chronic SIV and HIV infection (320-323). CTLs are excluded from the B cell follicle because they usually lack the follicular homing receptor CXCR5, which is not expressed until late in untreated infection (320). As a result, target cells within the follicle are protected from CTL-mediated cytolysis, whereas viral replication in EF zones is efficiently controlled (324). Indeed, ongoing SIV replication within T_{FH} cells of elite controller macaques has been attributed to CTL exclusion from B cell follicles, as depletion of SIV-specific $CD8^+$ T cells resulted in increased viral replication in non-T_{FH} $CD4^+$ T cells (54). In support of this finding, CD8 depletion in chronically infected rhesus macaques revealed greater increases in SIV RNA+ non-T_{FH} cells relative to T_{FH} cells (325). In addition to limitations of trafficking, some reports suggest that follicular $CXCR5^+CCR7^-$ $CD8^+$ T cells are less cytolytic than $CXCR5^-CCR7^-$ extrafollicular CTL. In SIV-infected rhesus macaques, follicular CTL frequently colocalized with regulatory T cells (T_{REG}) or expressed the exhaustion marker PD-1 (325). In human tonsils, approximately 90% of follicular $CD8^+$ T cells were characterized as $CD44^{hi}CXCR5^{hi}$ follicular

T_{REG} expressing IL-10, TGF-β, TIM-3, and low levels of perforin (326). This $CD44^{hi}CXCR5^{hi}$ subset was also shown to suppress T_{FH}-mediated immune responses to HIV infection *ex vivo* (326). However, other studies document that the rare $CXCR5^{+}CD8^{+}$ T cell population exhibits increased cytolytic potential compared to $CXCR5^{-}CD8^{+}$ T cells. For example, peripheral blood $CXCR5^{+}CD8^{+}$ T cells from HIV-infected individuals were characterized by higher levels of IFN-γ production and a less exhausted phenotype than EF $CD8^{+}$ T cells in a recent study by He *et al.* and inversely correlated with viral load (327). Furthermore, lymph node $CXCR5^{+}CD8^{+}$ T cells expressed higher levels of perforin compared to the $CXCR5^{-}CD8^{+}$ subset (327). Petrovas *et al.* confirmed this finding, but reported increased PD-1 expression and decreased polyfunctional cytokine responses in follicular $CD8^{+}$ T cells (311). In summary, the mechanisms governing CTL migration to and cytolytic activity within B cell follicles are poorly understood, and novel strategies to address barriers to CTL targeting of this compartment are being explored. Among these, temporary disruption of the B cell follicle with depleting antibodies (anti-CD20/rituximab), blockade of T and B cell interactions using anti-CD40L, and therapeutic vaccination with engineered CTL or chimeric antigen receptor T cells expressing CXCR5 have been proposed (54, 324). Transduction of CTL with CXCR5 to facilitate B cell follicle homing has been demonstrated in rhesus macaques (328). In addition, *in vitro* stimulation with TGF-β was able to induce CXCR5 expression in $CXCR5^{-}CD8^{+}$ T cells from chronically SIV-infected rhesus macaques (329). Besides their latency reversal properties, recombinant IL-15 and the IL-15 superagonist ALT-803 have both been shown to augment HIV-specific CTL responses and are thus being explored as enhancers of follicular CTL function (212, 324). Another promising approach involves the use of bispecific antibodies targeting CD3 and HIV gp120 that have the dual capacity to act as LRAs and to facilitate ADCC in gp120-expressing cells (330). Interestingly, the recently developed

CD3/VRC07 antibody was also shown to induce killing of HIV-infected cells by follicular CTL *ex vivo* (311). Although this remains to be tested *in vivo*, lymph node-homing $CXCR5^{+}CD8^{+}$ T cells could be transduced to produce these antibodies in order to facilitate killing of latently infected cells within the B cell follicle (324).

1.7 Dendritic cell programming for immunotherapy

Derived from the bone marrow, dendritic cells serve as bridge between the innate and adaptive immune systems due to their dual capacity to respond to pathogen- and danger-associated signals during acute inflammatory responses and to process and present antigens for the priming of naïve T cells (331). Although the principal human DC subsets *in vivo* consist of three main types (331), models of DC ontogeny have undergone numerous revisions since the discovery of DC by Ralph Steinman and Zanvil Cohn (332, 333) greater than 40 years ago. Due to recent experimental data supporting modification of the classical model of hematopoesis that regulates DC lineage, it is now accepted that DC originate not from a population of mutipotent progenitor cells but from progenitors that follow predestined developmental pathways determined by lineage priming (334-337).

DC are further classified based on differential expression of transcription factors such as interferon regulatory factors 4 and 8, which distinguish plasmacytoid DC (pDC, $IRF4^{+}IRF8^{+}$), myeloid/conventional DC1 (cDC1, $IRF4^{-}IRF8^{+}$), and myeloid/conventional DC2 (cDC2, $IRF4^{+}IRF8^{-}$) (331, 338-341). Characterized by surface expression of CD123 and CD45RA, pDC produce high levels of type I and type III interferons in response to viral infection in the forms of

single-stranded RNA and double-stranded DNA detected by endosomal toll-like receptors 7 and 9, respectively (331, 342, 343).

Myeloid cDC1 were initially classified as a subset of blood DC expressing high levels of CD141 (thrombomodulin) (344, 345) but also reside in numerous lymphoid and nonlymphoid tissues (331). Identification of cDC1 is also based on shared expression of CD13 and CD33 with cDC2 and low expression of CD11b, CD11c, and CD172 (331); unique to cDC1 is the receptor that recognizes actin exposed during cell necrosis, CLEC9A (346, 347). Myeloid DC1 have a superior capacity to cross-present viral and intracellular antigens in the context of MHC class I for activation of $CD8^+$ T cells and induction of T helper type 1 (T_H1) and natural killer (NK) cell responses (348-351). Like pDC, cDC1 produce both type I and type III interferons in response to pathogen sensing via TLR3 and TLR9 (331, 352-354).

The predominant human cDC population in blood, as well as lymphoid and nonlymphoid tissues, cDC2 are characterized by various surface antigens, including CD1c, CD2, CD172, FcεR1, CD11b, CD11c, CD13, and CD33 (331). Similar to monocytes, cDC2 express an extensive repertoire of lectins and pattern recognition receptors that facilitate immune responses to agents as diverse as intracellular pathogens, extracellular bacteria, parasites, and fungi (331). Furthermore, cDC2 are capable of inducing T_H1, T_H2, T_H17, and T_{REG} immune responses, due to their ability to secrete various pro- and anti-inflammatory mediators (331, 355-357). Of note, cDC2 are weak producers of type III interferons but can be stimulated to produce high levels of IL-12 compared to cDC1 (358-361).

Ex vivo generation and programming of DCs was first implemented in cancer immunotherapy to circumvent the dysfunction of endogenous DCs that occurs in cancer patients (362-367). In contrast to traditional vaccines that relied on antigen cross-presentation by patients'

own dysfunctional DCs, this strategy harnessed functional DCs generated outside of the tumor-associated suppressive environment to induce effective anti-cancer immunity (362-367). This concept was based on the premise that monocyte-derived DC could be imprinted during maturation with particular effector functions and homing properties for the induction of tumor-specific $CD8^+$ T cells *in vivo* (368). The first advantage of this approach is that *ex vivo*-generated DC can be loaded with specific antigens that will promote effective delivery of both antigen ('signal 1') and costimulation ('signal 2') to antigen-specific T cells. However, the ability of DC to cross-present antigen is affected by DC developmental stage and by the combination of factors used in their activation and maturation (369-373). In addition, shortcomings of 'first generation' DC vaccines highlighted the necessity for DC to express high levels of costimulatory molecules and lymph node homing capacity in order to induce effective anti-cancer CTL responses (368, 374-377). As a result, 'second generation' DC maturation strategies incorporating PGE_2 (378-380) were developed to induce fully mature DC with high expression of costimulatory molecules (CD83, CD86) and CCR7 (Table 2) (381-385). Although IL-1β/TNF-α/IL-6/PGE_2-matured DC displayed enhanced immunogenicity and migratory potential in response to lymph node-secreted chemokines CCL19 and CCL21 in healthy individuals (374-376, 381, 382), the negative impact of PGE_2 on DC IL-12p70 production (386-389) was linked to limited anti-tumor clinical responses (390).

Table 2. Immunotherapeutic potential of *ex vivo*-educated dendritic cells

Desired Trait	Current Standard (DC2)	Type 1-polarized DC (DC1)	References
Antigen presentation	++++	++++++	373
Maturation status (high CD83/CD86)	++++++	++++++	383, 385
Lymph node homing (CCR7)	++++++	++++	381-385
IL-12p70, IL-27, IL-23, IL-15 production	Deficient	++++++++ (superior inducers of Ag-specific T_H1 cells/CTL)	383, 385, 409
T cell trafficking/ chemokine expression	CCL22 (T_{REG})	CCL19 (T_N, T_{CM}) CCL3-5, CXCL9,10,11 (T_{EM})	384, 389, 417, 418
Antigen transfer ability	Deficient	++++++	385

Since high IL-12p70 production by DC has been shown to greatly enhance their ability to induce antigen-specific T_H1 cells and CTL ('signal 3') (391-401), subsequent efforts to improve the efficacy of DC-based cancer vaccines have focused on employing 'nonexhausted' DC, unlike those matured in the presence of IL-1β/TNF-α/IL-6/PGE_2 (380, 383, 402, 403). These 'type 1-polarized' DC (DC1) were initially generated in the presence of type I and type II interferons and TLR ligands, or IL-18-activated NK or memory $CD8^+$ T cells (383, 387, 404-410). Both *in vitro* and in mouse models, DC1 induced strong, long-lived tumor-specific CTL responses (368). Furthermore, addition of IFN-γ and LPS or a combination of IFN-γ, TNF-α, and IL-1β, to the cytokine maturation cocktail reversed maturation-associated DC exhaustion, producing polarized DC capable of increased IL-12p70 production in response to interaction with CD40L-expressing $CD4^+$ T cells (383, 387, 408, 411). DC-derived IL-15, IL-23, and IL-27 (Table 2), as well as DC-expressed intercellular adhesion molecule 1 (ICAM1) have also been identified as T_H1 cell-polarizing factors (409, 412, 413), whereas monocyte chemotactic protein 1 (MCP1, CCL2) and

OX40 ligand (OX40L) promote T_H2-type T cell responses (414). DC can also mediate regulatory T cell polarization through production of IL-10 and transforming growth factor-β (TGF-β) (415, 416).

Subsequent findings revealed that addition of IFN-α and the TLR 3 ligand polyinosinic:polycytidylic acid to maturing DC1 enhanced expression of CCR7 and production of chemokines ('signal 4') that promote interaction of DC1 with naïve, memory, and effector T cells (Table 2) (389). Specifically, inclusion of IFN-α in the maturation of these 'alpha type 1-polarized' DC (αDC1) augments production of CXCL9, CXCL10, CXCL11, and CCL5 to promote interaction of mature DC with CXCR3- and CCR5-expressing CTL, T_H1, and NK cells desirable for effector responses (384, 389, 417, 418). Likewise, IFN-α-induced maturation results in reduced production of CCL22 by αDC1, thus avoiding attraction of undesirable CCR4-expresssing T_{REG} (389).

In sum, while DC1 and PGE_2-matured DC (DC2) share similarities in antigen presentation, maturation status, and lymph node homing capacity, large discrepancies exist in their capacities for optimal cytokine production, chemokine expression related to T cell trafficking, and antigen transfer (Table 2). Thus, correct *ex vivo* programming is critical to the design of DC-based immunotherapies in order to achieve the desired cellular mechanisms *in vivo*. *Ex vivo* generation of DC allows manipulation of antigenic 'signal 1' and costimulatory 'signal 2' for optimal activation and expansion of both antigen-specific $CD4^+$ and $CD8^+$ T cells (368). Education of DC to further exploit polarizing 'signal 3' and homing 'signal 4' can selectively drive the desired antigen-specific T cell effector mechanisms and trafficking patterns. Importantly, this targeted combination strategy could improve therapeutic outcomes by circumventing logistic issues related to route of DC immunization, timely delivery of DC to the lymph nodes to minimize cytokine

exhaustion, and the requirement for large doses of DC to increase the chances of lymph node homing (374, 375, 384, 402, 403, 419-422).

1.8 Dual role for dendritic cells in the 'kick and kill'?

Historically, DCs have been implemented safely and successfully in clinical trials investigating cellular immunity to cancer (423), due to the critical concept of DC programming to achieve functional polarization (383, 385, 410). Several studies have shown that CD40L 'help' provided by $CD4^+$ T cells is a determining factor in the successful induction of antigen-specific CTL responses by DC through release of IL-12p70 (294, 383, 424). Consequently, the T cell priming capacity of DC is dependent upon their maturation and polarization status and responsiveness to CD40L signaling (383, 406, 425). However, the concept of DC polarization has yet to be adequately employed in DC-based HIV immunotherapies.

The gold standard PGE_2-matured DC (DC2) previously implemented in HIV clinical trials by our group (426) and others (427) are characterized by suboptimal responsiveness to CD40L resulting in a diminished capacity to induce primary CTL responses (294, 383), in addition to an exhausted cytokine phenotype (383, 385) and production of the T_{REG}-attracting chemokine CCL22 (389, 428). Conversely, the type 1-polarized DC (αDC1) that have achieved success in cancer immunotherapies are intentionally programmed to be superior drivers of type-1 cellular immune responses. Among their defining traits, αDC1 exhibit superior responsiveness to CD40L, an increased capacity for IL-12p70 production (383) and antigen processing (429, 430), chemokine secretion promoting desired T cell trafficking (384, 389), and the ability to induce effective primary CTL responses (383, 406, 407). Of note, CD40L-stimulated DC are able to prime HIV-

specific naïve $CD8^+$ T cells, thereby inducing the *de novo* CTL responses that are more effective than pre-existing memory CTL at killing virus-infected cells in ART-treated individuals during chronic infection (294). Furthermore, CD40L-induced IL-12p70 production is critical in this regard, as it 'licenses' DC to prime naïve T cells (431). Importantly, αDC1 are also able to 'reticulate' (385, 432), forming immune networks that facilitate intercellular transfer of antigenic information with lymph node-resident DC (433).

An early DC-based HIV immunization strategy developed by our group implemented autologous mature DC pulsed with HLA*A02-restricted HIV-1 Gag, Pol, and Env peptides and influenza A matrix protein peptide administered to participants intravenously or subcutaneously (426). Although the peptide-DC vaccine elicited HIV-specific IFN-γ responses at two weeks following the second immunization, the DC type used in this trial was functionally skewed towards the DC2 phenotype and thus suboptimal for the induction of long-lived, broadly reactive CTL responses. A subsequent study utilizing the same DC type and HIV-1/influenza peptide combination demonstrated an increase in the frequency of $CD4^+CD25^{hi}FOXP3^+$ T_{REG} cells post-vaccination that contributed to suppression of Gag-specific $CD8^+$ T cell polyfunctional cytokine responses (434). However, one of the most impressive HIV immunotherapy trials to date utilized DC pulsed with inactivated autologous HIV, which resulted in a 1 $\log_{10}$ decrease in HIV RNA setpoint and was associated with increased anti-HIV $CD8^+$ T cell IFN-γ responses (427). However, as with many previous DC-based studies, this trial implemented DC generation methods that yield IL-12p70-deficient DC incapable of inducing sustained HIV-specific effector responses. In an attempt to address this issue, Argos Therapeutics investigated *ex vivo* genetic manipulation of DC as a strategy to deliver a constitutive CD40L helper signal to the DC in an HIV immunotherapy to treat acute and chronic infections (435-437). Nevertheless, this approach was unsuccessful due to

the fact that constitutive CD40L signaling induces a burst of IL-12 production that ultimately creates IL-12p70-exhausted DC that are unresponsive to $CD4^+$ T_H cell interaction (294). A novel therapy proposed by Guardo *et al.* combined TRIMIX adjuvant and an HIV T cell immunogen (HTI) for *in vivo* targeting of DCs by intranodal injections (438). The previously described TRIMIX adjuvant consists of three mRNAs encoding CD40L, the costimulatory molecule CD70, and constitutively activated TLR4 (439). The HTI vaccine component consists of Gag, Pol, Vif, and Nef mRNA fragments chosen on the basis of antigen-specific $CD4^+$ and $CD8^+$ T cell reactivity (440). Monocyte-derived DC electroporated with this preparation were shown to induce T cell proliferation and IFN-γ responses *in vitro*, and intranodal injection of TRIMIX/HTI induced antigen-specific CTL responses in mice (438). In addition, human lymph node explants treated with TRIMIX/HTI activated DCs and induced proinflammatory mediator production. However, the IL-12-producing capacity of the mRNA/DC-based formulation was not investigated in this study, therefore providing no information regarding its potential to induce broadly reactive CTL required for the long-term control of viremia in the absence of ART (441).

Interestingly, results of a recent phase I/II clinical trial linked the administration of a DC-based HIV vaccine designed to induce CTL responses with increased residual viremia in ART-suppressed individuals following analytic treatment interruption (442). Comprised of DC loaded with HIV-1-infected apoptotic cells, this vaccine induced T cell activation, cytolysis of infected cells, and polyfunctional HIV-specific $CD8^+$ T cell cytokine responses a in a subset of participants. Although the vaccine did not prevent viral rebound during treatment interruption, the incidental findings suggest that the DC-based therapeutic acted as a LRA. However, that study was also not designed to specifically address the use of the DC therapeutic as an LRA, and a number of important questions remain, including the roles that DC polarization and antigen presentation

could have in the noted phenomenon, and the underlying mechanisms involved. Overall, although DC-based HIV-1 immunotherapies have proven to be safe and well tolerated, they have achieved a success rate of only 38% according to meta-analyses (443).

Although dendritic cells (DC) have been used in HIV-1 clinical trials for their capacity to induce antigen-specific T cell responses (426, 427, 444, 445), their HIV-1 LRA potential has been underexplored. Historically, most DC-based strategies aimed at the 'kick' of latent HIV have employed monocyte-derived immature DC (iDC) and DC matured by exposure to bacterial antigens (446-448). Among these studies, several have purported that DC-mediated LR could be induced by DC-T cell contact in the absence of antigen presentation and/or mediated by DC-secreted soluble factors. For example, van der Sluis *et al.* reported that interaction of monocyte-derived dendritic cells (MDDC) with actively proliferating primary T cells resulted in secretion of unidentified components by DC to induce latent provirus (446). However, in this study iDC were cultured with T cells that had been preactivated using either PHA or anti-CD3/CD28 beads. These treatments have been shown to reverse HIV-1 latency (72, 151, 222, 449, 450) and thus, it is difficult to dissect whether coculture with DC in this study impacted LR, since preactivation of the T cells alone could have induced proviral transcription. It has also been reported that HIV replication can be induced by stimulation of T cell surface molecules by antigen-presenting cells expressing costimulatory molecules such as CD40 and other B7 family members (451). For example, in an *in vitro model* of HIV-TB coinfection, upregulation of HIV-1 replication in alveolar macrophages required T cell contact as well as lymphocyte-derived cytokines (451). In this study, lymphocyte-macrophage contact resulted in downregulation of inhibitory CCAAT enhancer binding protein β and activation of NF-κB, facilitating proviral transcription through derepression of the HIV-1 LTR.

In yet another study, CD40L-transduced DC were reported to induce latency reversal in J-Lat and ACH-2 cell lines, independent of DC-T cell contact and mediated by TNF-α (452). Similarly, MDDC matured by AIDS-associated pathogens were shown to reactivate latent HIV in Jurkat T cells through secretion of TNF-α (448). Although the incorporation of antigen in DC-mediated LR has been underexplored, Marini *et al.* successfully utilized iDC loaded with SEB superantigen for reactivation of HIV-1 in *in vitro*–infected $CD4^+$ T cells (447). Finally, one recent study investigating the latency activation potential of various DC subsets demonstrated that tissue-resident and blood-derived myeloid DC reactivated virus *in vitro* from latently infected effector T cells with different efficiencies (453). Nevertheless, none of these DC therapeutics was designed with the dual purpose of creating sufficient HIV-1 antigen exposure for successful CTL targeting of the cellular reservoir.

In sum, the quality of DC implemented in HIV-1 immunotherapy trials to date is suboptimal to type-1 polarized DC. I hypothesize that under optimal conditions, a DC-based therapeutic strategy can be designed to safely facilitate both the 'kick' and 'kill' of the latent HIV-1 reservoir (Figure 1). I will show that clinically applicable, type 1-polarized, monocyte-derived DC (MDC1) are uniquely capable of both activating HIV-1 transcription in latently infected $CD4^+$ T cells harboring replication-competent virus and inducing broad HIV-1-specific CTL responses that can effectively target the exposed infected cells. To promote strong HIV-1-specific CTL responses, these antigen-presenting MDC1 are deliberately programmed to subsequently release high amounts of the critical CTL-inducing cytokine IL-12p70 upon interaction with the $CD4^+$ T helper cell factor CD40L (385). This is achieved using a combination of factors, including IFN-α, IFN-γ, and the TLR3 agonist poly (I:C), that are designed to mimic maturation events expected to occur as a result of DC crosstalk with responding IFN-α-producing pDC and IFN-γ-producing

NK cells during the initial phase of a successful antiviral immune response (383, 407). MDC1 have already been successfully implemented in clinical oncology trials (368, 396, 429, 454-456) due to their superior capacity to drive long-lived CTL responses from naïve T cell precursors (457, 458) and have recently been approved for use in phase I HIV clinical trials. I predict that the strategic inclusion of heterologous virus-associated antigens, designed to encourage such $CD4^+$ T cell 'helper' activity through MHC class II presentation, will also facilitate MDC1-mediated LR, thus offering a safe and directed means to immunologically expose and target the latent reservoir as part of a functional cure strategy for HIV-1 infection.

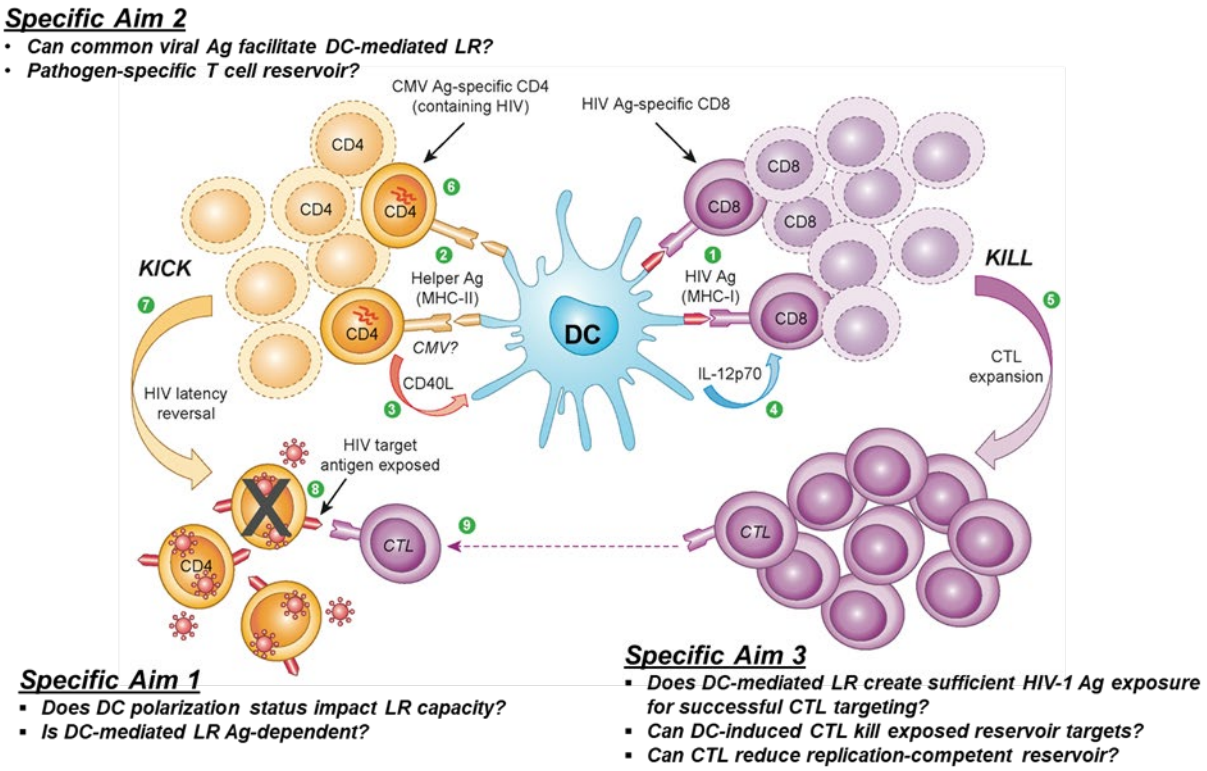


Figure 1. Proposed model of DC-mediated 'kick and kill'

DC can induce antigen-specific $CD8^+$ and $CD4^+$ T cell responses through presentation of antigenic peptides in the context of **(1)** MHC class-I and **(2)** MHC class-II molecules, respectively (signal 1) , along with costimulatory factors including CD80 and CD86 (not shown, signal 2). Responding $CD4^+$ T cells subsequently provide DC with the feedback hyperactivating 'helper' signal CD40L **(3)**, necessary for DC release of IL-12p70 **(4)**, which then promotes expansion and differentiation of $CD8^+$ antigen-specific effector CTL **(5)**. If the antigen-responsive $CD4^+$ T cells harbor latent HIV-1 **(6)**, their activation can result in HIV latency reversal **(7)**, with HIV-1 proteins being transcribed and expressed as surface antigen **(8)**. As a result, these exposed infected cells become targets for CTL. We hypothesize that MHC-class II presentation of CMV antigen will facilitate $CD4^+$ T cell 'help' for CTL induction, while exposing $CD4^+$ T cells harboring latent HIV-1.

2.0 SPECIFIC AIMS

2.1 AIM 1.1

Determine the impact of DC polarization status of antigen-presenting DC on the ability to induce HIV-1 latency reversal.

Hypothesis: If properly programmed, DCs will effectively induce HIV-1 latency reversal. In Aim 1.1, I will investigate the ability of differentially matured, monocyte-derived DC to induce *ex vivo* transcription of latent provirus in autologous $CD4^+$ T cells of virally suppressed individuals. I hypothesize that type 1-polarized, monocyte-derived DC (MDC1) will have a superior capacity to induce HIV-1 latency reversal compared to PGE_2-polarized, monocyte-derived DC (PGE_2-DC). Also referred to as αDC1 (383), MDC1 possess several characteristics that make them desirable therapeutic vaccine candidates. Among these, the combination of fully mature status, expression of high levels of costimulatory molecules, enhanced lymph node migratory potential, and increased IL-12p70 production compared to the 'gold standard' PGE_2-DC, has not been achieved by any DC-based vaccines (383). In addition, several cytokines produced by DCs, including IL-2 (459-462), IL-6, IL-7 (463, 464), and TNF-α, have been shown to be potent inducers of latency reversal in resting $CD4^+$ T cells of ART-treated individuals (465). DC-secreted IL-2 has also been reported to augment CD40/CD40L interaction (466), as well as serve as a possible 'signal 3' for T cells (462). Importantly, IL-2 produced by DCs has been detected in the immune synapse between T cells and DCs loaded with cognate antigen (467). As such, I further hypothesize that DC LRA activity will require antigen-driven interaction with $CD4^+$ T cells.

2.1.1 Aim 1.1a

Assess the impact of DC polarization and the requirement for antigen in the DC-mediated 'kick'.

2.1.2 Aim 1.1b

Utilize DC to generate outgrowth of autologous virus to be utilized as antigen for induction and assessment of HIV-specific CTL responses, as well as target cells for the assessment of CTL killing capacity (Aim 3).

2.1.3 Aim 1.1c

Determine if bidirectional signaling through CD40L impacts DC-mediated latency reversal.

2.2 AIM 1.2

Determine the impact of DC polarization status on the ability to induce HIV-1-specific CTL. Hypothesis: If optimally programmed, DCs will induce HIV-1-specific CTL. Mailliard *et al.* first demonstrated the efficacy of type 1-polarized DC as anticancer vaccines due to their expression of functional characteristics critical for induction of long-lived antigen-specific CTL (383). In addition, a recent study determined that novel HIV-1-specfic CTL responses could be selectively induced from naïve $CD8^+$ T cells of chonic HIV-infected individuals on suppressive cART by priming with autologous virus-loaded type 1-polarized DC (294). This study also reported that

HIV-specific memory $CD8^+$ T cell responses were dysfunctional, resulting in IFN-γ secretion but suboptimal effector function (294). However, the type 1-polarized and PGE_2-matured DC types have never been directly compared for their ability to prime HIV-specific CTL responses. As a proof of concept, in Aim 1.2 I will utilize autologous $CD8^+$ T cells of HLA-$A2^+$ HIV-naïve individuals to investigate the efficacy of differentially polarized MDC1 and PGE_2-DC to induce HIV-1-specific CTL.

2.2.1 Aim 1.2a

Assess the impact of DC polarization on CTL priming capacity.

2.2.2 Aim 1.2b

To determine whether CD40L 'help' during priming impacts the quality of HIV-1-specific CTL responses. The DC type with higher CTL priming capacity (as determined in Aim 1.2a) will be implemented in this aim.

2.3 AIM 2

Utilize common viral antigens to facilitate DC unveiling of the latent HIV-1 reservoir within $CD4^+$ T cells having antigen specificity to these viruses.

Hypothesis: Inclusion of viral (CMV, Influenza, HIV) heterologous MHC Class II helper antigens will promote interaction of DC with $CD40L^+$ T helper (T_H) cells and facilitate exposure of the

latent reservoir hidden within these CD4 compartments for immune elimination. For a DC-based LRA to be clinically applicable, antigen other than SEB will be required to facilitate DC-mediated activation of latently infected $CD4^+$ T cells. However, it is unclear whether an antigen-specific association with the HIV-1 cellular reservoir exists. Elucidating which antigen(s) to use would allow for a more directed DC-based targeting of antigen-specific $CD4^+$ T cells harboring the virus, rather than a global T cell activation approach. I propose that inclusion of the aforementioned common viral antigens as a component of our DC-based therapeutic will promote interaction with CD40L-expressing $CD4^+$ T_H cells, for the dual purpose of providing immune 'help' for DC-mediated induction of HIV-1-specific CTL responses and facilitating DC-mediated exposure of viral antigen-specific $CD4^+$ T cells harboring latent HIV, to simulate a clinically relevant method of HIV-1 latency reversal. The DC type determined to be most effective at mediating both the 'kick' and the 'kill' in Aim 1 will be implemented in this aim.

2.3.1 Aim 2a

Assess the efficacy of clinically relevant, common viral antigens in facilitating DC-mediated latency reversal.

2.3.2 Aim 2b

Utilize common viral antigens for DC-mediated generation of autologous target cells to be utilized for evaluation of CTL killing capacity (Aim 3).

2.4 AIM 3

Determine the efficacy of MDC1-mediated latency reversal in enhancing susceptibility of the HIV-1 reservoir to immune targeting.

Hypothesis: An optimal DC-mediated latency reversal strategy will create sufficient HIV-1 antigen exposure for successful targeting of the reservoir by autologous CTL. We have recently shown that MDC1 are effective inducers of HIV-1 specific CTL, the effector cell type associated with early control of HIV infection (468). However, for CTL to 'kill' requires proper surface antigen expression on target cells. Based on the efficacy of MDC1 in inducing HIV-specific CTL responses, I will utilize our MDC1-based priming model (294, 383, 418) to generate CTL biosensors for use in determining if our DC-based LRA approach exposes the HIV-1 reservoir for susceptibility to CTL 'kill'. I propose that proviral antigen presented by latently infected $CD4^+$ T cells will be recognized by MDC1-primed autologous CTL, resulting in targeted killing of these cells. Furthermore, I will demonstrate that our MDC1-based approach can also facilitate specific immune elimination of those cells harboring replication-competent provirus.

2.4.1 Aim 3a

Evaluate effector function of MDC1-primed autologous CTL.

2.4.2 Aim 3b

Demonstrate HIV-specific killing of DC-induced target cells generated in Aims 1b and 2b by MDC1-primed autologous CTL in short-term cytotoxicity assays.

2.4.3 Aim 3c

Demonstrate suppression of viral outgrowth by MDC1-primed autologous CTL in long-term cytotoxicity assays.

2.4.4 Aim 3d

Assess the ability of MDC1-primed autologous CTL generated in Aim 3a to eliminate latently infected cells harboring replication-competent provirus.

3.0 MATERIALS AND METHODS

3.1 Study participants

HIV-1-infected ART-treated participants of the Pittsburgh clinical site of the Multicenter AIDS Cohort Study (MACS) were selected for this research. These participants were documented as having begun ART with a median virally controlled treatment duration of 12.3 years (range 1.7 – 20.8 years; Table 3). Whole blood products from HIV-1-negative blood donors were purchased from the Central Blood Bank of Pittsburgh. Written informed consent was obtained from participants prior to inclusion in the study. The University of Pittsburgh Institutional Review Board approved this study.

Table 3. Characteristics of study participants

Participant ID	Age (years)	Time to treatment (years)	Time on suppressive ART (years)	ART regimen*	Viral load* (copies/mL)	$CD4^+$ T cell count* ($cells/mm^3$)
1	65	0.5	1.7	TUM	< 20	1191
2	67	6.0	20.8	EFV + CBV	< 20	702
3	77	0	13.2	RTV + RTVB	< 20	1007
4	56	1.7	10.5	ATP	< 20	973
5	58	5.3	17.8	ABC + CPR	32	476
6	56	0.6	17.5	ATP	< 20	779
7	58	5.4	10.7	RTV + RTVB + ATZ + TRU + TUM	< 20	936
8	53	6.8	7.9	ATZ + TRU + IST + DCV	< 20	351
9	47	8.25	14.2	ABC + LPV + TDF + RTVB	< 20	489
10	49	2.7	14.2	ATP	< 20	686
11	52	5.25	12.25	EFV + EPZ	< 50	721
12	55	2.2	11.8	ATP	< 20	434
13	56	6.8	18.25	LAM + RTV + ABC	< 20	334
14	55	3.4	3.8	CPR + ODS	ud	852
15	51	7.5	11.2	TZV	< 20	706
16	71	15.9	12.8	NVP + TRU	< 20	535
17	69	6.25	10.0	ATP	< 20	263

Asterisk (*), at visit; ud, undetectable. ABC, abacavir; ATP, atripla; ATZ; atazanavir; CBV, combivir; CPR, complera; DCV, descovy; EFV, efavirenz; EPZ, epzicom; IST, isentress; LAM, lamivudine; LPV, lopinavir; NVP, nevirapine; ODS, odefsey; RTV, ritonavir; RTVB, ritonavir boosted; TDF, tenofovir disoproxil fumarate; TRU, truvada; TUM, triumeg; TZV, trizivir.

3.2 Isolation of monocytes and peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) obtained from buffy coat or whole blood were isolated by standard density gradient separation using Lymphocyte Separation Medium (Corning, Manassas, VA). PBMC were further separated into monocytes and peripheral blood lymphocytes (PBL) using a positive selection human CD14 microbeads kit (Miltenyi Biotec, Auburn, CA) according to manufacturer's specifications, and the differentially isolated cells were cryopreserved until use.

3.3 Generation of monocyte-derived DC

Immature DC were generated from monocytes isolated and cultured for 5 days in Iscove's Modified Dulbecco's Media (IMDM) containing 10% fetal bovine serum and 0.5% gentamicin in the presence of granulocyte-monocyte colony-stimulating factor (GM-CSF; 1000 IU/mL; Sanofi-aventis, Bridgewater, NJ) and interleukin-4 (IL-4; 1000 IU/mL; R&D Systems, Minneapolis, MN). As previously described (385), mature, high IL-12p70-producing MDC1 and IL-12p70-deficient, prostaglandin E_2-treated DC (PGE_2-DC) were generated by exposure of immature DC at day 5 for 48 hours to a cocktail of maturation factors containing either interferon (IFN)-α (1000 U/mL; Schering Corporation, Kenilworth, NJ), IFN-γ (1000 U/mL; R&D Systems, Minneapolis, MN), IL-1β (10 ng/mL; R&D Systems, Minneapolis, MN), tumor necrosis factor (TNF)-α (25 ng/mL; R&D Systems, Minneapolis, MN), and polyinosinic:polycytidylic acid (20 ng/mL; Sigma-Aldrich,

St. Louis, MO), or IL-1β (10 ng/mL), TNF-α (25 ng/mL), IL-6 (1000 U/mL; R&D Systems, Minneapolis, MN), and PGE_2 (2 μM; Sigma-Aldrich, St. Louis, MO), respectively.

3.4 Flow cytometry

Phenotypic characterization of DC was determined by flow cytometry using cells stained with the following antibodies: CD14-PE (clone TÜK4; Miltenyi Biotec, Auburn, CA), CD83-PE (clone HB15A; Beckman Coulter, Brea, CA), CD86-PE (clone HA5.2B7; Beckman Coulter, Brea, CA), CCR7-FITC (clone 150503; R&D Systems, Minneapolis, MN), OX40L-PE (clone ik-1; BD Biosciences, San Jose, CA), Siglec-1/CD169-Alexa Fluor® 488 (clone 7-239; Bio-Rad, Hercules, CA), CD209-APC (clone DCN46; BD Biosciences), and HLA-DR-APC-Cy7 (clone L243; Biolegend, San Diego, CA). For surface staining, cells were preincubated with 1X PBS labeling buffer containing 2% BSA, 0.1% NaN3, and unfractionated murine IgG (1.0 μg/mL; Sigma-Aldrich) to block Fc-receptor binding. $CD4^+$ T cells cocultured with MDC1 were tested weekly for the presence of HIV-1 p24 by surface staining for CD3 (APC-H7, clone SK7; BD Biosciences) and CD4 (Pacific Blue, clone RPA-T4; BD Biosciences), and intracellular staining with KC57-FITC antibody (clone FH190-1-1, Beckman Coulter). Antigen-specific CTL responses were assessed by exposing CTL to HIV-1 Gag 9-mer peptides (1 μg/mL) or media alone, and incubating with CD107a-FITC (clone H4A3; BD Biosciences) stain mix containing 0.1% monensin (BD Golgi Stop™, BD Biosciences) for six hours at 37º C. Cells were then stained for viability (LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Life Technologies), surface expression of CD3 (APC-H7, clone SK7; BD Biosciences) and CD8 (PerCP-Cy5.5, clone SK1; BD Biosciences), and intracellular expression of IFN-γ (IFN-γ-AlexaFluor® 700, clone B27; BD Biosciences).

3.5 Functional characterization of differentially matured DC

DC production of IL-12p70 in response to CD40L-transfected J558 cell (J558-CD40L; a gift from Dr. P. Lane, Birmingham, UK) stimulation was determined as previously described (418). Briefly, DC were plated (2.5 x 10^4 cells/well) in a 96-well flat-bottom plate and stimulated with J558-CD40L (5 x 10^4 cells/well) for 24 hours. Culture supernatants were collected and tested by IL-12p70 ELISA using the following reagents: Recombinant Human IL-12 Standard (R&D Systems), Primary Human IL-12 mAb (Thermo Scientific, Watham, MA), Secondary Human IL-12 mAb, Biotin-labeled (Thermo Scientific), HRP-conjugated Streptavidin (Thermo Scientific), TMB Substrate Solution (Thermo Scientific), Stop Solution (Thermo Scientific).

3.6 Induction of HIV-1 LR in $CD4^+$ T cells

MDC1 were tested for their ability to induce HIV-1 LR by coculture with autologous $CD4^+$ T cells in the absence or presence of SEB (Sigma-Aldrich), CMV pp65 (CMVpp65 Recombinant Protein or PepTivator® CMV pp65, Miltenyi Biotec), HIV-1 Gag (HIV-1 IIIB PR55 Gag Recombinant Protein, NIH AIDS Reagent Program; HIV-1 Gag Recombinant Protein, Sigma-Aldrich; or HIV-1 Consensus 15-mer Peptides, Sigma-Aldrich), or influenza M1 antigen (Influenza M1 Protein (A/California/04/2009) (H1N1), eEnzyme, or PepTivator® Influenza A (H1N1) MP1, Miltenyi Biotec). Briefly, total $CD4^+$ T cells were isolated from cryopreserved PBL derived from HIV-1-infected MACS participants by negative magnetic bead separation using an EasySep™ Human $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies, Cambridge, MA). $CD4^+$ T cells were cocultured with DC in complete IMDM at a ratio of either 1:7 (100,000 DC: 750,000 $CD4^+$ T

cells) or 1:10 (100,000:1 x 10^6) for seven days in 48-well plates. Total $CD4^+$ T cells from HIV-1-infected MACS participants were treated with Dynabeads® Human T-Activator CD3/CD28 (Life Technologies, Carlsbad, CA) and implemented as a positive control in LR experiments. The cytokines rhIL-2 (Proleukin®, 100 U/mL; Prometheus Laboratories Inc., San Diego, CA) and rhIL-7 (1 µg/mL; Miltenyi Biotec, Auburn, CA) were added to the cultures on day 4, and culture supernatants were harvested on day 7 for quantitation of HIV-1 RNA. Where stated, cocultures were maintained and the T cells tested for intracellular expression of p24 on days 15-20 by flow cytometry. CD40L blocking antibody (clone MK13A4, 10 µg/mL; Enzo Life Sciences, Farmingdale, NY), Leaf™ Purified Mouse IgG1,k isotype antibody (clone MG1-45, 10 µg/mL; Biolegend), and efavirenz (300nM; Cayman Chemical, Ann Arbor, MI) were used where shown.

3.7 Relative Quantification of HIV-1 Gag RNA

Culture supernatants were ultra-centrifuged (Sorvall Stratos Biofuge) at 45,000 x g for 1 hour at 4º C to obtain viral pellets from which total RNA was isolated by the RNA-Bee™ method (TEL-TEST, Inc., Friendwood, TX). Five microliters of RNA were used for detection of reverse transcription using TaqMan® Reverse Transcription Reagents (Life Technologies) according to the manufacture's protocol. A 20 µL TaqMan® PCR was performed by mixing 5 µL cDNA with TaqMan® Universal PCR Master Mix (Thermo Fisher), 500nM each of forward (5'-CCCATGTTTTCAGCATTATCAGAA-3') and reverse primers (5'-CCACTGTGTTTAGCATGGTGTTTAA-3'; Integrated DNA Technologies, San Jose, CA), and 250nM FAM/MGB-labeled probe (5'-FAM-AGCCACCCCACAAGA-MGB-3'; Integrated DNA Technologies). Real-time PCR was performed using the using the ViiA 7 A&B Applied

Biosystems instrument (Life Technologies) and the following cycling conditions: 50º C for 2 min, 95º C for 10 min, 40 cycles of 95º C for 15 sec, and 60º C for 1 min. Real-time PCR primers and probes were based on the HIV-1 pNL4.3 sequence encoding the *gag* region. Serially diluted pNL4.3 plasmid DNA ranging from 10^1 to 10^6 copies applied to each PCR assay served as the HIV-1 standard curve. A no template control was included in each assay to control for PCR cross-contamination, and each sample was assayed in triplicate. QuantStudioTM Real-time PCR Software (Applied Biosystems, Foster City, CA) was used for PCR data analysis and copy number estimation.

3.8 Generation and characterization of HIV-1-infected $CD4^+$ T cell targets

Total $CD4^+$ T cells cocultured with antigen-presenting MDC1 and were tested weekly for the presence of HIV-1 p24 antigen by intracellular flow cytometry staining with KC57-FITC antibody (clone FH190-1-1, Beckman Coulter). Target cells were pre-screened for p24 expression, and cryopreserved for later use as targets in CTL killing assays when they reached at least 10% positivity.

3.9 Induction and expansion of autologous CTL

Total $CD8^+$ T cells were isolated from cryopreserved PBL by negative magnetic bead separation using an EasySep™ Human $CD8^+$ T Cell Enrichment Kit (STEMCELL Technologies). To induce CTL responses as previously described (418), $CD8^+$ T cells were cocultured with autologous

differentially matured DC loaded with either HLA-A2-restricted Gag p24 ($Gag_{151–159}$) 9-mer peptide epitopes when using HIV-1-negative blood donors, or Gag p17/p24 overlapping 15-mer peptides (1 μg/mL, Sigma-Aldrich) when using HIV-1-infected MACS participants. The cocultures (75,000 DC: 750,000 $CD8^+$ T cells) were treated with or without the addition of either 25,000 gamma-irradiated (5000 rad) CD40L-transfected J588 cells (J558-CD40L; a gift from Dr. P. Lane, Birmingham, UK) (469) or rhCD40L (0.25 μg/mL; Enzo Life Sciences) where stated. On day 5, rhIL-2 (250 U/mL) and rhIL-7 (10 ng/mL) were added to the cultures and every three days thereafter. On day 12, T cell cultures were restimulated with either gamma-irradiated HLA-$A2^+$ T2 cells (for induction of primary CTL responses in HLA-$A2^+$ HIV-1-negative donors) or differentially matured autologous DC loaded with autologous 9-mer peptides (1 μg/mL) corresponding to the viral antigens and DC type used in the initial stimulation. Antigen-specific readout assays were performed between days 20-24 to assess CTL activity.

3.10 IFN-γ ELISPOT assays

Autologous CTL (3-5 x 10^4/well) were tested for reactivity to individual and pooled Gag 9-mer peptide antigens (1-10 μg/mL) by ELISPOT assay using anti-human IFN-γ and biotin monoclonal antibodies (clones 1-D1K and 7-B6-1; Mabtech, Cincinnati, OH) as previously described (294, 418). Recorded values were net responses compared to control wells consisting of CTL exposed to assay medium alone.

3.11 HIV-1-infected cell killing assays

CTL effector function was assessed as described previously, with modifications (294). Briefly, MDC1-stimulated total $CD8^+$ T cells were cocultured with autologous DC-induced $CD4^+$ target cells at various effector:target (E:T) ratios for 18 hours at 37º C. Harvested cocultures were stained for surface expression of CD8 (PerCP-Cy5.5, clone SK1; BD Biosciences) and intracellular expression of HIV-1 p24 (KC57-FITC, clone FH190-1-1; Beckman Coulter). Effector $CD8^+$ cells were excluded from analysis gating, and the percent reduction in infected $CD4^+$ T cells was determined at each E:T ratio. For colorimetric cytolytic assays, autologous CD4 cells were stained with either CFSE (eBioscience, San Diego, CA) or CellTrace™ Violet (Thermo Fisher) dyes following the manufacturer's protocols. Target cells (CFSE) were then loaded with individual peptides at 100 ng/mL in PBS for 60 min at room temperature (RT); excess unbound peptide was removed by washing. The CFSE and CellTrace™ Violet dye-labeled cells were mixed in equal numbers and coincubated for 18 hours with autologous CTL at various E:T ratios. The antigen-specific killing of HIV-1 peptide-loaded $CD4^+$ T cells (green) was calculated based on relative changes in percentages of the differentially stained target cells remaining, using flow cytometry analysis.

3.12 Viral outgrowth assays

Total $CD8^+$ T cells were cocultured with autologous p24-expressing $CD4^+$ target cells at various E:T ratios as described for the CTL kill assay. Cultures were maintained for eight days, after which

culture supernatants were harvested and tested by p24 ELISA (Frederick National Laboratory for Cancer Research, Frederick, MD) for CTL-induced viral suppression (418).

3.13 Quantification of replication-competent HIV-1

Culture supernatants harvested from LR and viral outgrowth assays were spinoculated onto TZM-bl cell (NIH AIDS Reagent Program) monolayers (30,000 cells/well) for four hours at 300 g and cocultured for 48 hours. Beta-Glo® reagent (Promega, Madison, WI) was added to PBS-washed TZM-bl cell monolayers and incubated for 1 hour at room temperature. Control supernatants from cultured $CD4^+$ T cells of an uninfected donor were treated in parallel. Chemiluminescence from the TZM-bl cells was detected by luminometer as previously described (72). Sample wells were considered positive for the presence of replication-competent virus if the chemiluminescent signal exceeded the mean + 2 S.D. of a control sample.

3.14 Statistical analyses

Statistical analyses for ELISPOT and ELISA data were calculated using a linear mixed model with 95% confidence intervals and Wilcoxon matched-pairs signed-ranks test, respectively. Differences between MDC1-mediated LR (HIV-1 RNA) were determined by multilevel mixed-effects tobit regression analyses or Wilcoxon matched-pairs signed-ranks test. Criteria for inclusion in latency reversal experiments were $\geq$ 30 copies/mL of viral RNA in the anti-CD3/CD28 positive control group and $\geq$ 20 copies/mL of viral RNA in all other experimental

groups. For the purpose of statistical analysis, values < 20 copies/mL of viral RNA were assigned a value of 20 copies/mL.

4.0 RESULTS

4.1 MDC1 effectively induce primary HIV-1-specific CTL responses with CD40L 'help'

Successful CTL targeting of the latent HIV-1 reservoir will require recognition of HIV-1-associated peptide epitopes presented on infected cells upon LR. Besides latency itself, major hurdles for effective CTL elimination of HIV-1 infected cells include issues related to $CD8^+$ T cell exhaustion (470), alterations in CTL epitopes, antigen processing, and antigen presentation associated with immune escape (471, 472); the establishment of epitope variants that act as partial agonists to induce dysfunctional noncytolytic cross-reactive memory CTL responses (294, 418), and presentation of target antigen decoys by cells harboring defective virus (295). Together, these points highlight the need to generate highly functional CTL either through induction of *de novo* $CD8^+$ T cell responses, or subdominant memory responses targeting relevant conserved epitopes of the reservoir-associated virus.

We initially compared the use of two clinically applicable, differentially activated DC preparations using blood products from HLA-$A2^+$ HIV-1-naïve blood bank donors to test their capacity to induce primary HIV-1-specific CTL responses. As previously described (385), mature type 1-polarized monocyte-derived DC (MDC1) and monocyte-derived DC matured in the presence of PGE_2 (PGE_2-DC) were generated for comparison. MDC1 are characterized by their mature phenotypic status (Figure 2A) and high capacity to produce IL-12p70 upon subsequent stimulation with the $CD4^+$ T cell 'helper' signal CD40L, while PGE_2-DC are IL-12p70-deficient and less responsive to CD40L signaling (Figure 2B, C) (385).

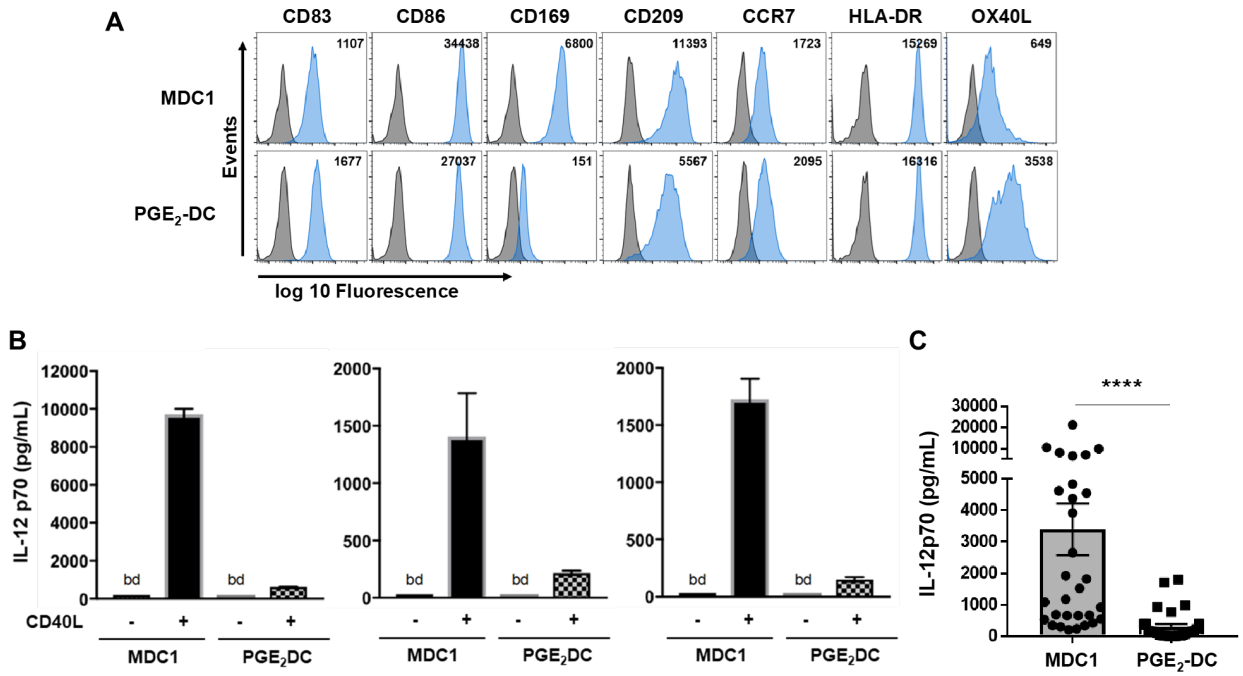


Figure 2. MDC1 are programmed to produce enhanced levels of IL-12p70 upon stimulation with CD40L

A) Differentially polarized mature DC were analyzed for surface phenotype. Gray histogram peaks of flow cytometry plots indicate unstained control samples; peaks shaded in blue represent positive staining for the phenotypic markers indicated. Inset numbers refers to MFI. **B)** Mature DC were plated (2.5 x 10^4 cells/well) in a 96-well flat-bottom plate and cultured in presence or absence of J558-CD40L (5 x 10^4 cells/well) for 24 hours. Culture supernatants were collected and tested by IL-12p70 ELISA. Error bars indicate mean ± S.D.; n = 3. bd = below detection limit of the assay (37pg/ml). **C)** Mature DC were tested for their net IL-12p70-producing capacity above background in response to CD40L stimulation. *P* values were determined by Wilcoxon matched-pairs signed-ranks test. Error bars indicate mean ± SEM; n = 30. ****P<0.0001.

Furthermore, MDC1 express significantly higher levels of sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1, CD169) than PGE_2-DC (Figure 3A) (unpublished, in preparation), which facilitates capture and transfer of HIV-1 virions to $CD4^+$ T cells in a process termed *trans* infection (473, 474). However, because our proposed MDC1-based immunotherapy is intended for *in vivo* administration during ART, the potential for *trans* infection of susceptible $CD4^+$ T cells is negligible. Interestingly, the high level of Siglec-1 expression and other adhesion molecules may also contribute to the extensive 'stickiness' of MDC1, which is evident in the morphological differences seen when culturing MDC1 under standard adherent (Figure 3B, left panel) vs. low-binding (Figure 3B, right panel) culture conditions (unpublished, in preparation). We have found that the use of the low-binding culture surfaces has no impact on the functional properties of the MDC1, but this strategy does greatly enhance the yield (Figure 3C) and viability (Figure 3D) of these cells when they are cultured in the absence of serum, and therefore may be a useful approach for culturing MDC1 for therapeutic application.

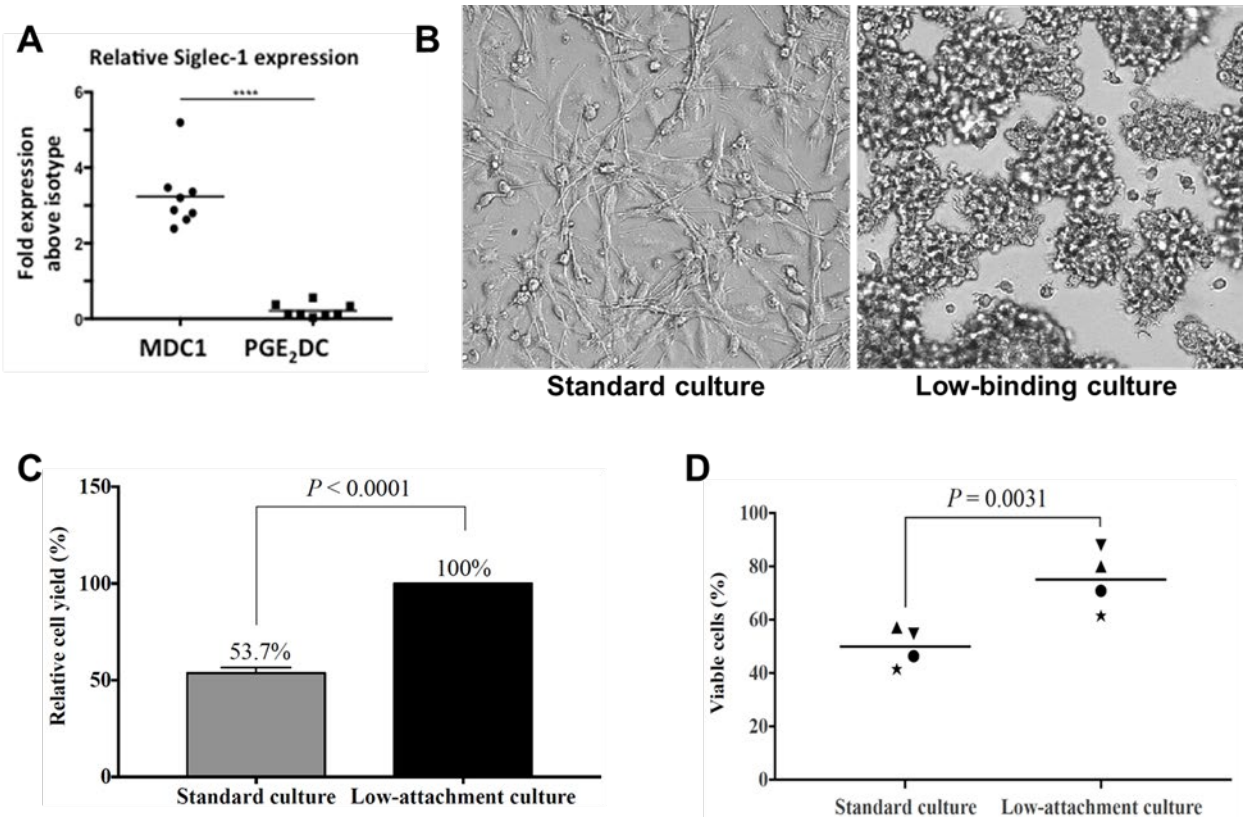


Figure 3. Impact of Siglec-1 expression on MDC1 morphology

A) Relative expression of Siglec-1 compared to isotype control staining on MDC1 and PGE_2-DC generated from healthy donors (n=8). *P* values were determined by Wilcoxon matched-pairs signed-ranks test. Error bars indicate mean ± SEM; ****P<0.0001. **B)** Bright-field images (original magnification 200X) of MDC1 standard culture (left panel) and low-binding culture (right panel), representative of 5 independent experiments from MDC1 of 4 healthy donors. **C)** Relative recovery of MDC1 from standard and low-attachmnet cultures. **D)** Viability of MDC1 cultured under standard and low-attachment conditions.

MDC1 also possess the unique ability to 'reticulate' (385), forming F-actin-based cellular extensions known as tunneling nanotubes (TNTs) in response to the $CD4^+$ T_H signal CD40L (Figure 4A, left panel) (385). Blockade of the CD40/CD40L signaling axis abrogates TNT formation and significantly decreases IL-12p70 production (Figure 4A, right panel; Figure 4B) (Zaccard *et al*., in submission). Absent in PGE_2-DC (385), TNTs serve as intercellular communication networks for the transfer or exchange of ions and electrical signals, proteins, organelles, and antigenic information between cells (385, 475-480). Of significance, TNTs have also been implicated in the transmission of pathogens, including HIV-1 (480-483).

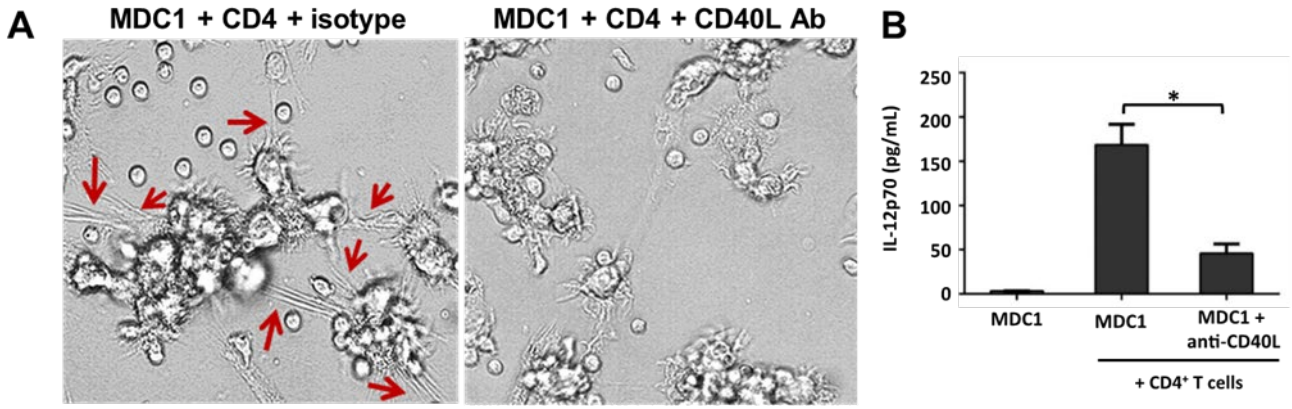


Figure 4. Influence of CD40L blockade on MDC1 morphology and function

A) MDC1 were cocultured with activated $CD4^+$ T cells in the absence (left panel) or presence (right panel) of CD40L blocking antibody for 24 hours prior to imaging. Bright-field images (400X) were enhanced to permit visualization of CD40L-induced membrane extensions and nanotube networks. **B)** Supernatants collected from MDC1 and MDC1/$CD4^+$ T cell cocultures in the absence or presence of CD40L blocking antibody were tested for IL-12p70 content by ELISA. *P* values were determined by Wilcoxon matched-pairs signed-ranks test. Error bars indicate mean ± SEM; **P*<0.05.

MDC1 and PGE_2-DC were loaded with HLA-A2-restricted HIV-1 peptide antigen and used as *in vitro* stimulators of autologous $CD8^+$ T cells, in the presence of gamma-irradiated (5000 rad) CD40L-expressing J558 cells (J558-CD40L), which served as a $CD40L^+$ T_H cell surrogate. In doing so, we found that MDC1 had a higher CTL priming capacity compared to PGE_2-DC (Figure 5A, B) (424). Importantly, the effective *in vitro* induction of long-term CTL responses by MDC1 required the presence of CD40L 'help' during the initiation of the priming cocultures (Figure 5C).

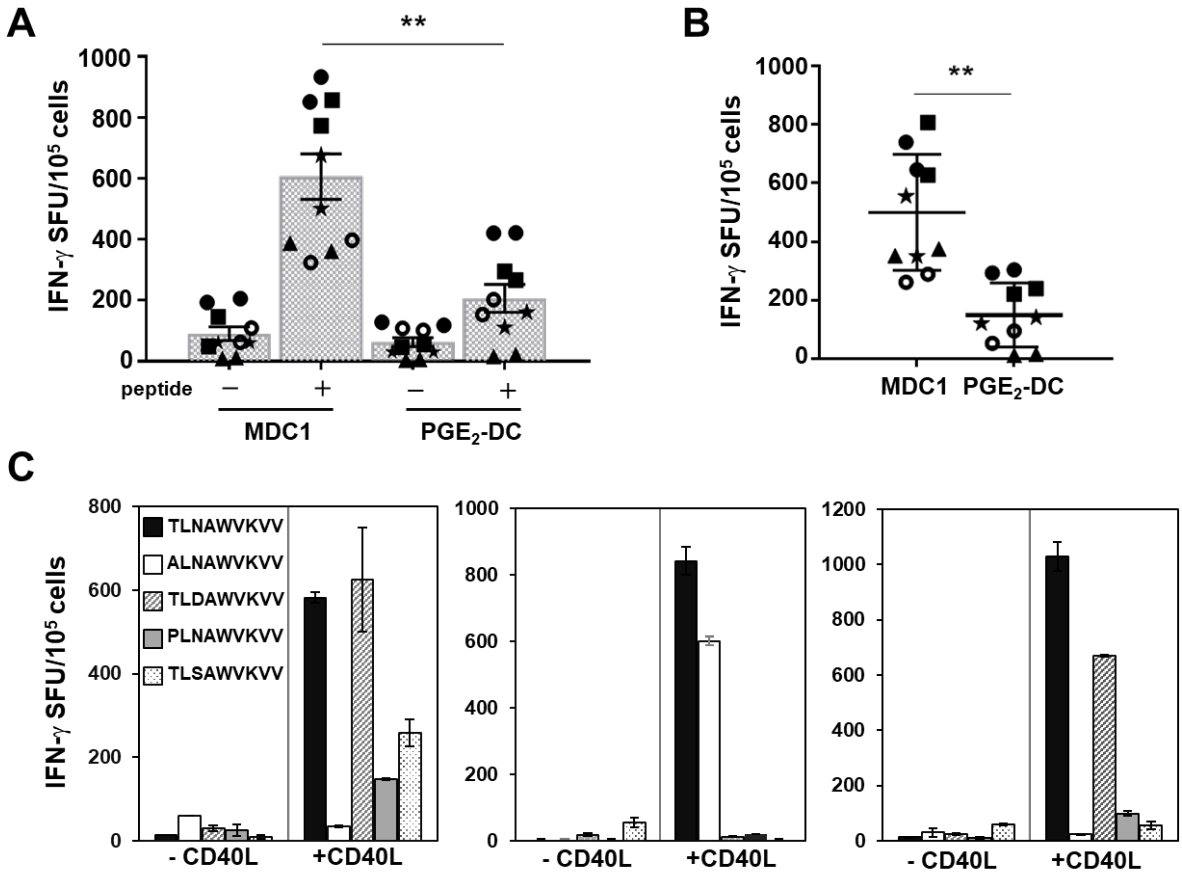


Figure 5. MDC1 are superior inducers of HIV-1-specific CTL responses

A, B) MDC1 and PGE_2-DC loaded with HIV-1 $Gag_{151-159}$ peptide (TLNAWVKVV) were cocultured with autologous $CD8^+$ T cells of HLA-A2+ HIV-1-naïve individuals for expansion and characterization of antigen-specific CTLs by IFN-γ ELISPOT. Shown are **A)** IFN-γ responses in the absence and presence of peptide stimulation and **B)** net values above subtracted unstimulated background. *P* values were calculated using a linear mixed model with 95% confidence intervals. Error bars indicate mean ± S.D. ***P*<0.01. **C)** IFN-γ ELISPOT results of $CD8^+$ T cell responses to $Gag_{151-159}$ peptide variants induced in 3 different HIV-uninfected donors by autologous antigen-presenting MDC1 in the absence or presence of CD40L.

4.2 Antigen presentation by autologous DC drives HIV-1 latency reversal in $CD4^+$ T cells

Recent evidence linked a DC therapeutic with an increase in residual HIV-1 viremia while the study participants were on ART, suggesting that the DC therapeutic acted in some way as an LRA (484). However, the mechanisms involved in this phenomenon are not yet clear, including the role that antigen presentation may have played. Although MDC1 were shown to be strong inducers of primary CTL responses (Figure 5), their efficacy as an LRA has not previously been explored. As such, we next compared the capacities of MDC1 and the 'gold standard' PGE_2-DC to induce HIV-1 latency reversal.

In preliminary experiments, MDC1 and PGE_2-DC were generated from ART virally suppressed HIV-1-infected MACS participants and cocultured with autologous peripheral blood $CD4^+$ T cells in the presence or absence of SEB antigen (Figure 6A). SEB was used because as a superantigen, it can effectively facilitate immune cross-talk between antigen-presenting cells and a large percentage (~30%) of SEB-responsive T cells (385). $CD4^+$ T cells treated with anti-CD3/CD28 mAb-coated beads were used as a positive LRA control (151). These experiments revealed the inherent difficulty in reactivating latent provirus from $CD4^+$ T cells of virally suppressed individuals with variable reservoir sizes and $CD4^+$ T cell counts. Foremost, the large volumes of blood (250 mL) or leukapheresis that were required for the isolation of monocytes and peripheral blood lymphocytes needed to perform the experiments were a considerable limitation of this study. Due to the fact that 3 to 5 million $CD4^+$ T cells per experimental condition were needed for latency reversal experiments, CD4 recovery from peripheral blood lymphocytes often determined the number of experimental conditions investigated and whether experiments could be repeated if necessary. Furthermore, the timing of viral RNA detection post-stimulation also varied widely between study participants. For example, when culture supernatants were tested

longitudinally for HIV RNA, inducible provirus did not become detectable at the same time points in all participants (Figure 6B) and was noninducible in others. When comparing MDC1 + SEB and PGE_2-DC + SEB stimulation and choosing HIV RNA > 100 copies/mL as a criterion, viral RNA in culture supernatants became detectable between 5 and 19 days post-stimulation. Levels of inducible provirus varied widely between donors, ranging from 127 to 32,191 copies/mL (Figure 6B).

Comparing the impact of MDC1/Ag vs. PGE_2-DC/Ag stimulation over time, we determined that although comparable (data not shown), the highest levels of HIV RNA became detectable at earlier time points in a greater number of participants with MDC1-mediated LR (Figure 6C). For example, MDC1/Ag stimulation induced the greatest 'kick' in eight participants at day 5 post-stimulation, whereas PGE_2-DC/Ag stimulation produced this effect in only one donor at day 5. In an effort to control for this vast donor variability, we chose to screen participants for their ability to respond to maximum anti-CD3/CD28 stimulation (38, 72), and selected only those individuals in which LR could be detected by day 7 post-culture for inclusion in the study. Accordingly, HIV RNA values obtained only at day 7 post-stimulation were used as a cutoff to assess DC-mediated LR in all subsequent experiments.

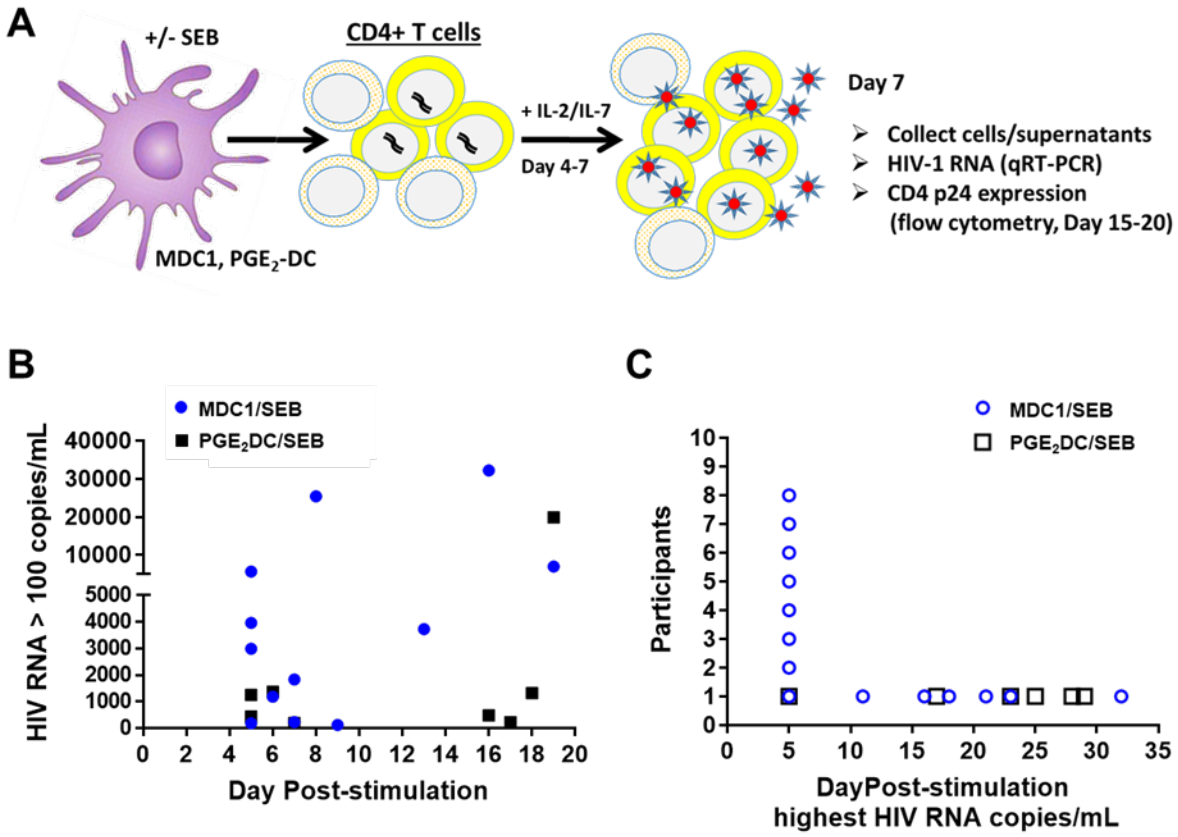
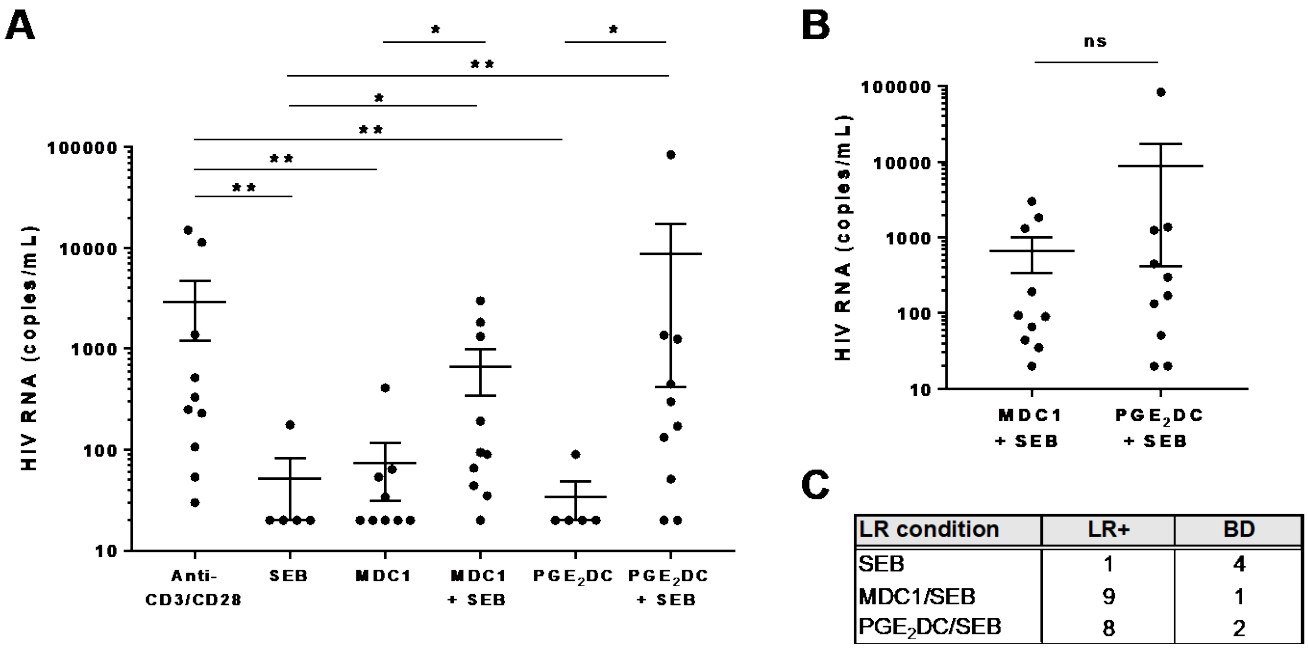


Figure 6. Timing of DC-mediated HIV-1 latency reversal is highly variable among participants

A) Model of DC-mediated HIV-1 latency reversal in autologous $CD4^+$ T cells. **B)** Differentially polarized DC were cocultured with autologous $CD4^+$ T cells in the presence or absence of SEB antigen. Cell culture supernatants were analyzed by qRT-PCR for HIV-1 RNA. Data points indicate day post-stimulation at which HIV RNA reached > 100 copies/mL. **C)** Differentially polarized DC were cocultured with autologous $CD4^+$ T cells in the presence or absence of antigen. Cell culture supernatants were analyzed by qRT-PCR for HIV-1 RNA. Shown are the days post-stimulation at which the highest HIV RNA values were detected.

Using these criteria, qRT-PCR analysis of HIV-1 RNA presence in day 7 coculture supernatants revealed that MDC1 indeed acted as a strong LRA in an SEB antigen-dependent manner (Figure 7A; MDC1 alone vs. MDC1 + SEB, $P < 0.05$), as did PGE_2-DC (PGE_2-DC alone vs. PGE_2-DC + SEB, $P < 0.05$) (424). As expected, significant differences in transcription of proviral DNA were also observed between positive (anti-CD3/CD28) and negative (SEB) experimental controls ($P < 0.01$) and between SEB vs. DC + SEB conditions (SEB vs. MDC1 + SEB, $P < 0.05$; SEB vs. PGE_2-DC, $P < 0.01$). No significant differences in LRA activity between anti-CD3/CD28 and DC + SEB stimulation or between SEB and DC alone conditions were detected. Importantly, MDC1 and PGE_2-DC also did not differ in their abilities to mediate antigen-dependent HIV-1 latency reversal (Figure 7B), which becomes most apparent when normalizing the data based on mRNA expression levels above (LR+) or below the limit of detection (BD) by qRT-PCR (Figure 7C) (424).



LR condition	LR+	BD
SEB	1	4
MDC1/SEB	9	1
PGE_2DC/SEB	8	2

Figure 7. Influence of antigen presentation on DC-mediated HIV-1 latency reversal in $CD4^+$ T cells

A, B) Differentially polarized DC were cocultured with autologous $CD4^+$ T cells in the presence or absence of SEB antigen. Cell culture supernatants were analyzed by qRT-PCR for HIV-1 RNA at day 7. *P* values comparing viral RNA levels were determined by multilevel mixed-effects tobit regression analyses. Error bars indicate mean ± SEM. **P*<0.05 and ***P*<0.01. C**)** Latency reversal scores based on data represented in (A). LR+, latency reversal positive; BD, below limit of detection.

However, to be clinically relevant, latency reversal strategies in HIV-infected individuals must be implemented under the administration of ART in order to prevent new infection events. As a proof of principle we repeated the DC/SEB-mediated LR experiments conducted in Figure 4 in the presence or absence of the nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV). NNRTIs inhibit reverse transcription through an allosteric mechanism that involves noncompetitive binding to HIV-1 reverse transcriptase (RT) at a site adjacent to the DNA polymerase active site (485). qRT-PCR analysis for both cell-associated (Figure 8A) and extracellular virion-associated (Figure 8B) HIV-1 RNA showed increased MDC1/SEB LRA activity compared to PGE_2-DC/SEB reactivation, which was reproduced in the presence of EFV, albeit at lower levels. These findings indicate that DC-mediated LR is achievable in the presence of antiretroviral therapy. Furthermore, because MDC1 exhibited both higher CTL priming capacity (Figure 5) and the ability to reactivate latent HIV-1, this DC type was implemented in Aims 2 and 3 for the dual purpose of exposing latently infected cells for subsequent CTL targeting.

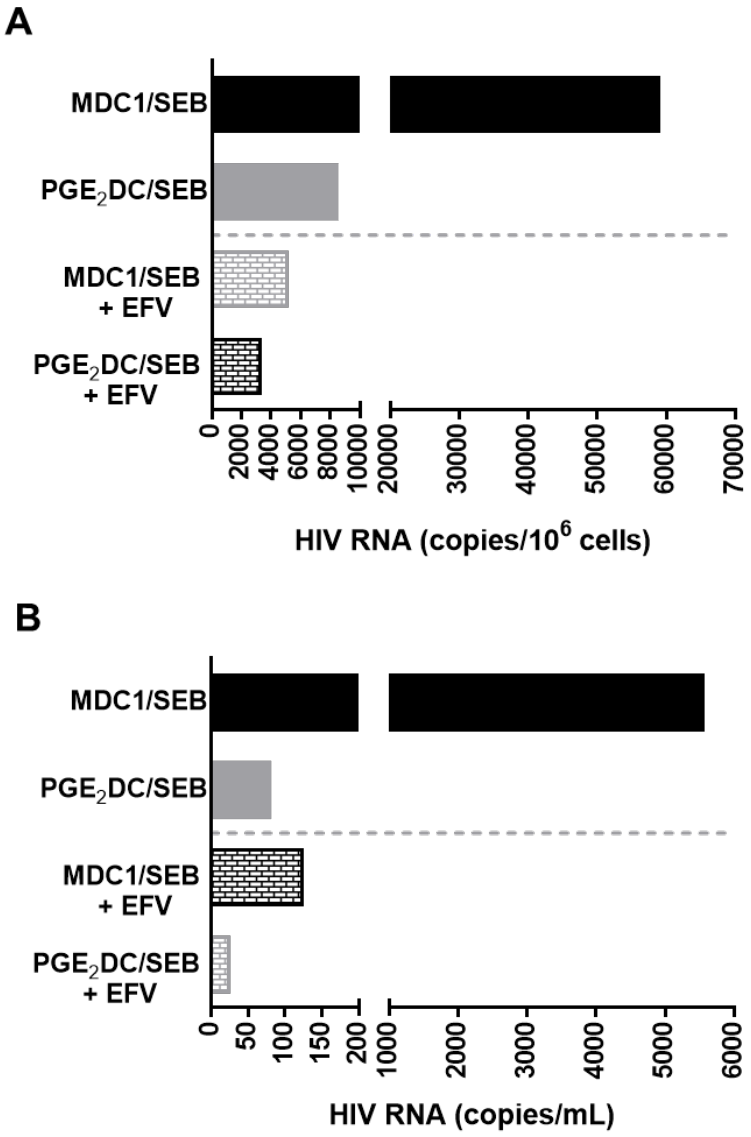


Figure 8. Antigen-loaded DC facilitate HIV-1 latency reversal in the presence of ART

SEB-loaded, differentially polarized DC were cultured with autologous $CD4^+$ T cells in the presence or absence of efavirenz. **A)** Cells and **B)** cell culture supernatants were analyzed by qRT-PCR for HIV-1 RNA at day 7. Representative of one donor.

4.3 Role of CD40/CD40L interaction in MDC1-mediated 'kick' of latent HIV-1

DC crosstalk with $CD40L^+$ T_H cells plays a critical role in the induction and survival of long-term CTL responses (458, 486-488). Because we previously showed that MDC1 are particularly sensitive to CD40L signaling (385), and that this $CD4^+$ T cell-derived 'helper' factor is required for effective MDC1-mediated *in vitro* priming of *de novo* CTL responses (Figure 5B) (424), we wanted to determine if CD40/CD40L cross-talk between the MDC1 and $CD4^+$ T cells was playing a role in MDC1-mediated HIV-1 latency reversal. Of note, CD40:CD40L ligation is required for optimal induction of polarizing signals (signal 3) that promote development of either T_H1 or T_H2 cells capable of pathogen-specific immune responses through triggering of NF-κB signaling (489-492). As the PKC agonist and disulfiram classes of pharmacological LRAs operate through this mechanism to induce transcription of latent HIV-1 (155, 171, 178, 179, 493-496), we reasoned that CD40:CD40L interaction might play a role in our model of MDC1-mediated latency reversal. Indeed, we found that blocking CD40/CD40L interaction strongly decreased the effectiveness of MDC1-mediated LR (424). The impact of this CD40L signaling inhibition on MDC1-mediated LR was clearly evident when analyzing the activated $CD4^+$ T cells by flow cytometry, where the addition of an anti-CD40L blocking antibody resulted in a marked inhibition of CD4 downregulation (87.7% ± 3.1%, $P < 0.05$; Figure 9A and B), a phenomenon that occurs with HIV-1 protein translation (497). As expected, this inhibition of CD4 downregulation by addition of the CD40L blocking antibody was associated with abrogation of intracellular p24 expression (90.8% ± 7.0%) (Figure 9A, C; $P < 0.05$) induced in autologous $CD4^+$ T cells, and with the reduction in HIV-1 RNA content in day 7 coculture supernatants measured by qRT-PCR (Figure 9D, 94.1% ± 6.1% inhibition) (424). Importantly, the addition of an isotype control antibody to the MDC1/T cell cocultures had no significant impact on the induced changes in CD4 expression or HIV-1

expression resulting from LR (Figure 9A-D). Taken together, these data support the required involvement of cognate antigen-driven bidirectional signaling events between MDC1 and antigen-responsive $CD4^+$ T cells in HIV-1 LR.

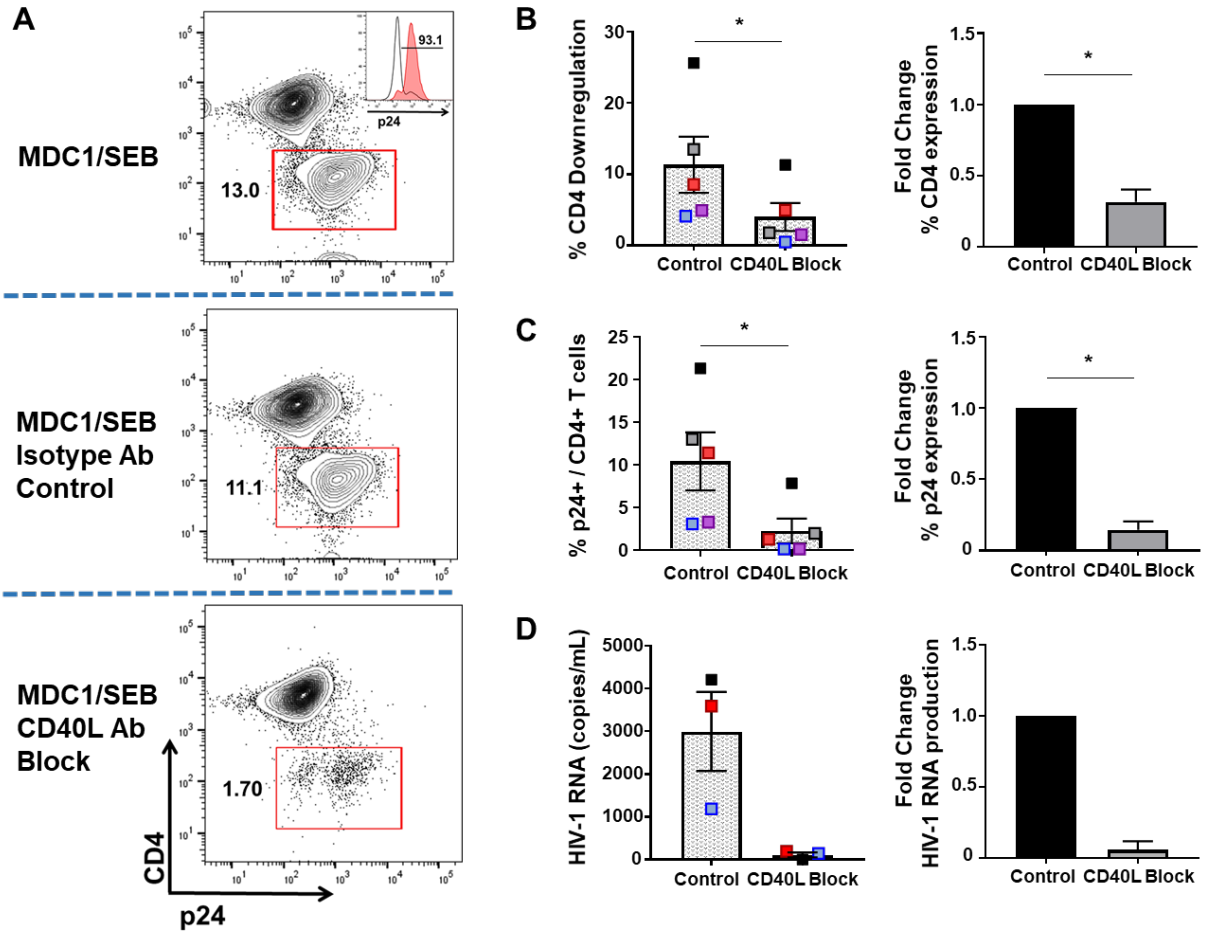


Figure 9. MDC1-mediated latency reversal requires CD40/CD40L interaction

DC1 were cocultured with autologous $CD4^+$ T cells and SEB to induce HIV-1 LR, in the presence or absence of CD40L blocking antibody. **A)** Representative flow cytometry plot of day 15 cultures. Downregulation of CD4 expression (red gate) corresponds with increased expression of p24 in the absence of CD40L blockade. Red histogram peak corresponds with p24 expression of CD4 downregulated population. **B, C)** Graphical representation of CD4 downregulation **(B)** and p24 expression **(C)** of populations described in (A); n = 5. **D)** Day 7 cell coculture supernatants were analyzed by qRT-PCR for HIV-1 RNA; n = 3. Differences between MDC1/SEB-mediated LR in the absence or presence of CD40L blocking antibody were compared by Wilcoxon matched-pairs signed-ranks test. Error bars indicate mean ± SEM. **P*<0.05.

4.4 CMV and HIV-1 antigen-driven reactivation of latent HIV-1 by MDC1

We have shown that MDC1-mediated transcription of HIV-1 DNA (Figure 7) and subsequent translation of p24 (Figure 9A, C) are both dependent on the presence of SEB superantigen and on CD40/CD40L signaling (424). However, to simulate a clinically relevant method of HIV-1 LR, we posited that the inclusion of common viral MHC class II antigens as part of our MDC1-based therapeutic could promote interaction with CD40L expressing $CD4^+$ T helper (T_H) cells, to both provide immune 'help' for MDC1-mediated induction of HIV-1-specific CTL responses and to facilitate MDC1-mediated exposure of viral antigen-specific $CD4^+$ T cells harboring latent HIV-1.

In choosing which viral antigens to incorporate in our model of MDC1-mediated LR, we considered previous findings that a significant pool of latently infected $CD4^+$ T cells are HIV-1-specific (498, 499). As such, an HIV-1-based vaccine or LRA construct could potentially reactivate this population while also facilitating the 'kill' through CTL priming. We also considered the fact that approximately 95% of HIV-1-infected individuals are coinfected with CMV (500), in whom CMV-specific $CD4^+$ T cell memory inflation occurs (501), with some having greater than 25% of their T cells specific to CMV (502). To test these findings in our cohort of MACS participants, we cocultured CMV antigen-loaded MDC1 with autologous peripheral blood lymphocytes (PBL) for expansion and characterization of CMV-specific T cell responses by flow cytometry (Figure 10). Indeed, at baseline there was already a substantial number of T cells responsive to CMV antigen that could be generated by non-specific culture expansion (6.38%), and a striking percentage of CMV-specific T cells that could be specifically expanded (36.9%) with CMV antigen-presenting MDC1, based on coexpression of CD107a and IFN-γ. Therefore, we hypothesized that inclusion of heterologous CMV antigen would effectively promote MDC1

interaction with $CD4^+$ T_H cells to facilitate 'help' for HIV-1-specific CTL induction and to induce CMV antigen-specific MDC1-mediated LR.

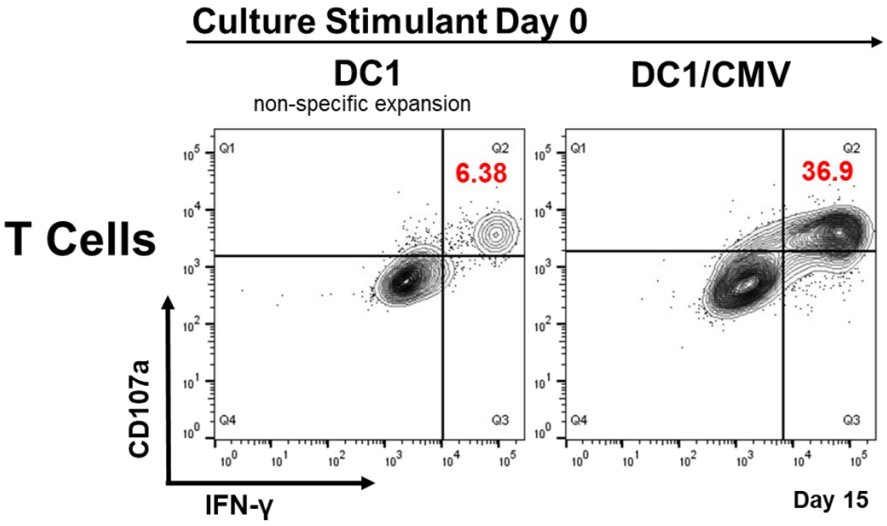


Figure 10. Large percentage of T cell repertoire in HIV^+ individuals is CMV-specific

MDC1 alone or MDC1 loaded with CMV pp65 15-mer peptides were cocultured for 15 days with autologous PBL for expansion and characterization of antigen-specific CTLs. Peptide restimulated cells were assayed by flow cytometry for expression of CD107a and IFN-γ.

We tested MDC1 alone or loaded with CMV pp65, HIV-1 Gag, or influenza A virus M1 protein (representing a common, non-persistent viral antigen) for their ability to induce latency reversal in autologous $CD4^+$ T cells (Figure 11). We found that MDC1-mediated LR was antigen-dependent, with CMV and HIV-1 antigen having notable LR activity, while influenza virus antigen did not (Figure 11B-E) (424). MDC1/CMV- and MDC1/HIV-1-mediated increases in extracellular virion-associated HIV-1 RNA were significantly greater than those induced by MDC1 alone (Figure 11B; $P < 0.01$ and $P < 0.001$, respectively). Importantly, MDC1 presenting either CMV or HIV-1 antigen exposed latent HIV-1 cellular reservoir targets, identified by a marked downregulation in CD4 expression (Figure 11D) that corresponded to increases in intracellular p24 (Figure 11C, E) (424).

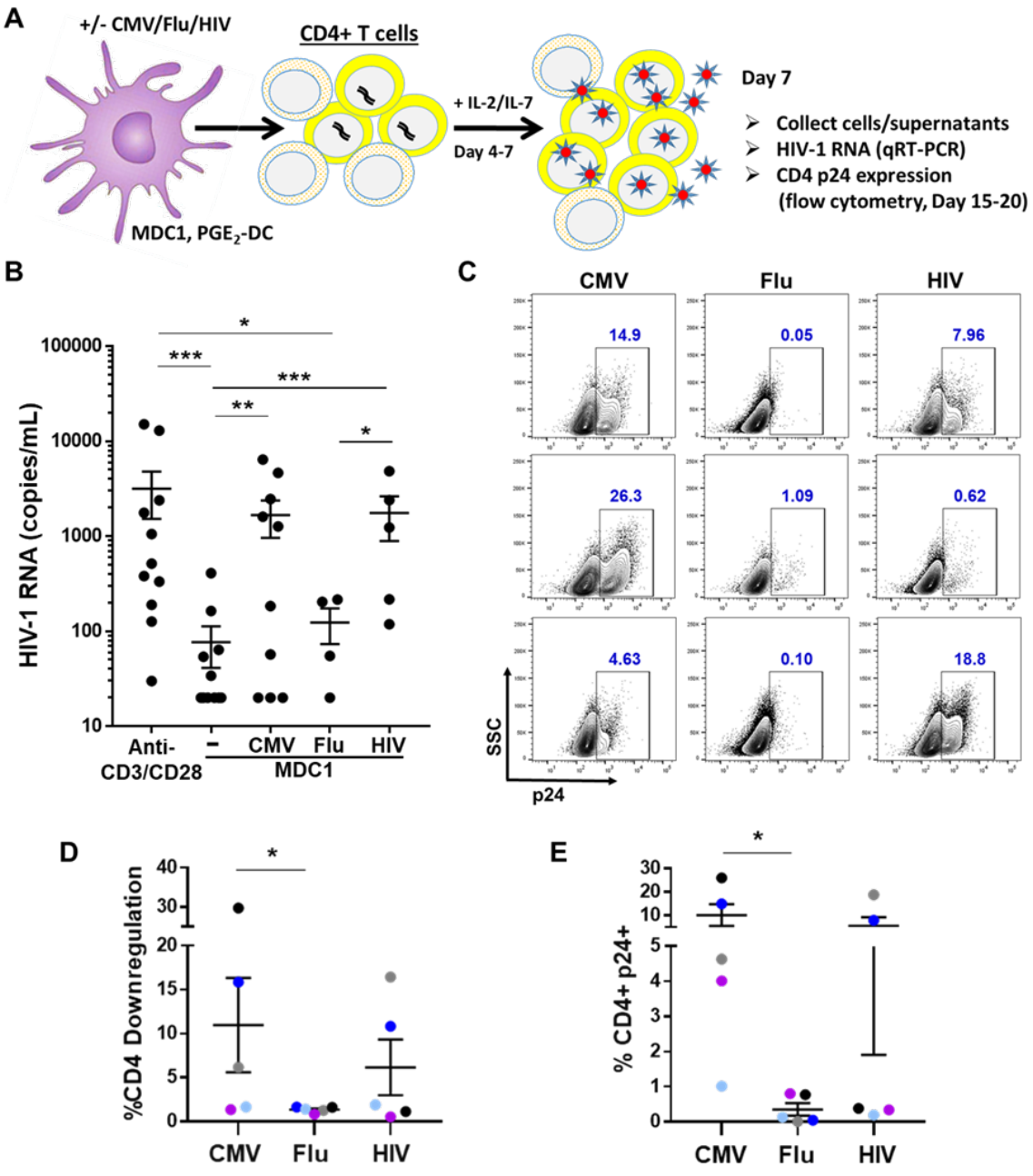


Figure 11. CMV and HIV antigen presentation drive MDC1-mediated HIV-1 latency reversal

A) MDC1 were loaded with either CMV pp65, HIV-1 Gag, or influenza M1 antigen and tested for their ability to induce LR in autologous $CD4^+$ T cells. **B)** Culture supernatants were assayed by qRT-PCR for detection of HIV-1 RNA at day 7. **C)** Representative flow cytometry plots of p24 expression of day 20 cocultures, gated on total $CD4^+$ T cells. **D)** Graphical representation of MDC1/antigen-induced CD4 downregulation in cocultures described in **(C)**, as measured by flow cytometry. **E)** Expression of p24 expression in cell populations detailed in **(C)**. *P* values comparing viral RNA levels were determined by multilevel mixed-effects tobit regression analyses. Error bars indicate mean ± SEM. **P*<0.05, ***P*<0.01, and ****P*<0.001.

4.5 MDC1-induced CTL effectively kill MDC1-exposed $CD4^+$ T cell targets harboring replication-competent HIV-1

MDC1 loaded with autologous HIV-1 Gag peptides were used to induce antigen-specific CTL (Figure 12A), as determined by flow cytometry analysis for antigen-induced expression of CD107a and IFN-γ (Figure 12B) and by IFN-γ ELISPOT (Figure 12C). Antigen-induced downregulation of CD8 expression, a characteristic previously shown to be associated with enhanced cytolytic capacity (418, 503), was evident along with high expression of CD107a and IFN-γ in the CTL generated *ex vivo* using HIV-1 antigen-presenting MDC1 (Figure 12B) (424). Also, the CTL responses induced by MDC1 were broadly reactive to a range of individual Gag 9-mer epitopes by IFN-γ ELISPOT (Figure 12C) (424).

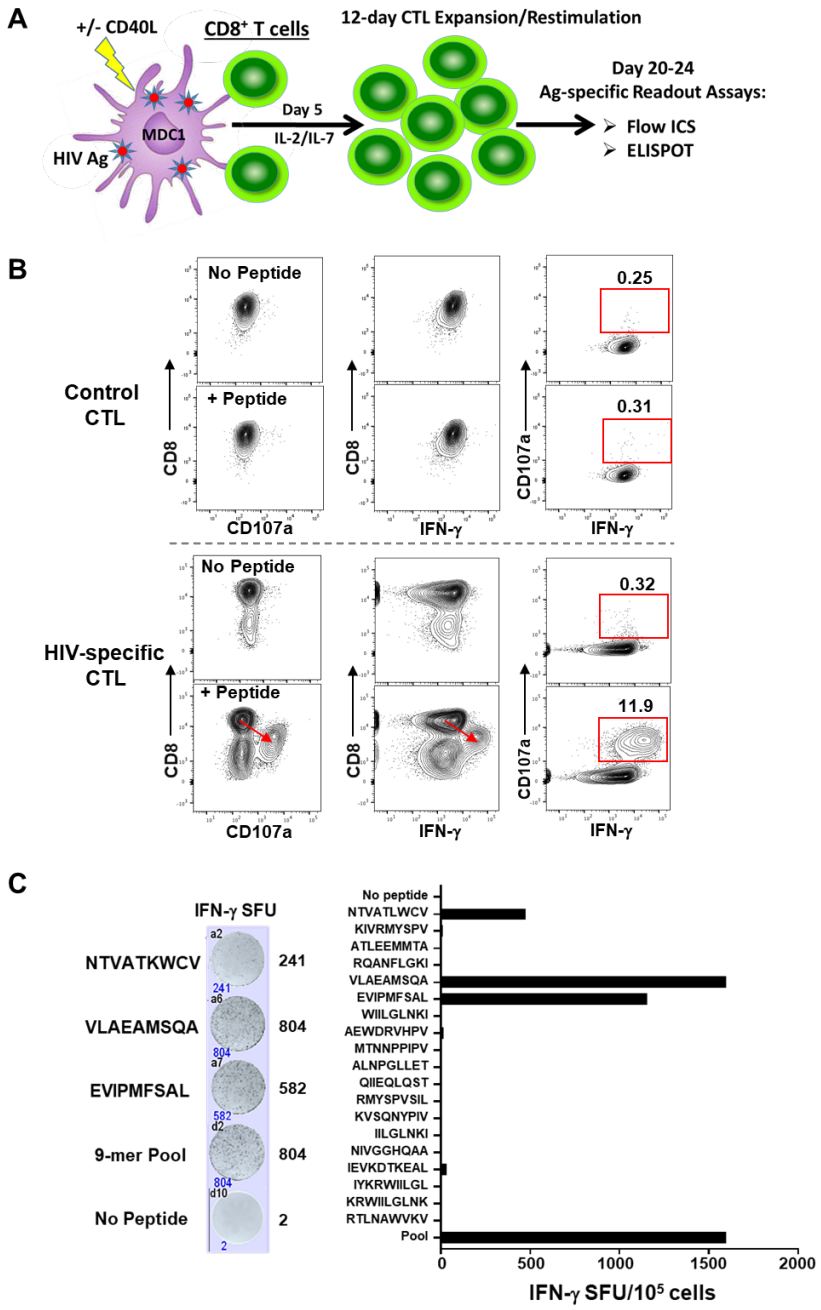


Figure 12. MCD1 induce broadly reactive HIV-1-specific CTL

A) MDC1 priming model for generation of autologous HIV-1-specific CTL. MDC1 loaded with HIV-1 Gag p17/p24 overlapping 18-mer peptides were cocultured with autologous $CD8^+$ T cells in the presence of IL-2 and IL-7, with or without the addition of either CD40L-transfected J588 cells or rhCD40L. T cell cultures were restimulated with autologous MDC1 loaded with 9-mer peptides corresponding to the viral antigens used in the initial stimulation. CTL activity was assessed by antigen-specific readout assays between days 20-24. **B)** MDC1 induced broadly reactive antigen-specific autologous CTL as determined by flow cytometry staining for CD107a and IFN-γ. **C)** Polyclonal IFN-γ responses to individual Gag 9-mer epitopes by MDC1-induced CTL described in **(B)**.

Next, HIV-1 Gag peptide-loaded CD4$^+$ T cells or p24-expressing target cells generated in Aims 1.1b and 2b by MDC1-mediated latency reversal were cocultured with the HIV-specific autologous CTL at increasing effector-to-target ratios to test for antigen-specific targeting of the latent reservoir (Figure 13).

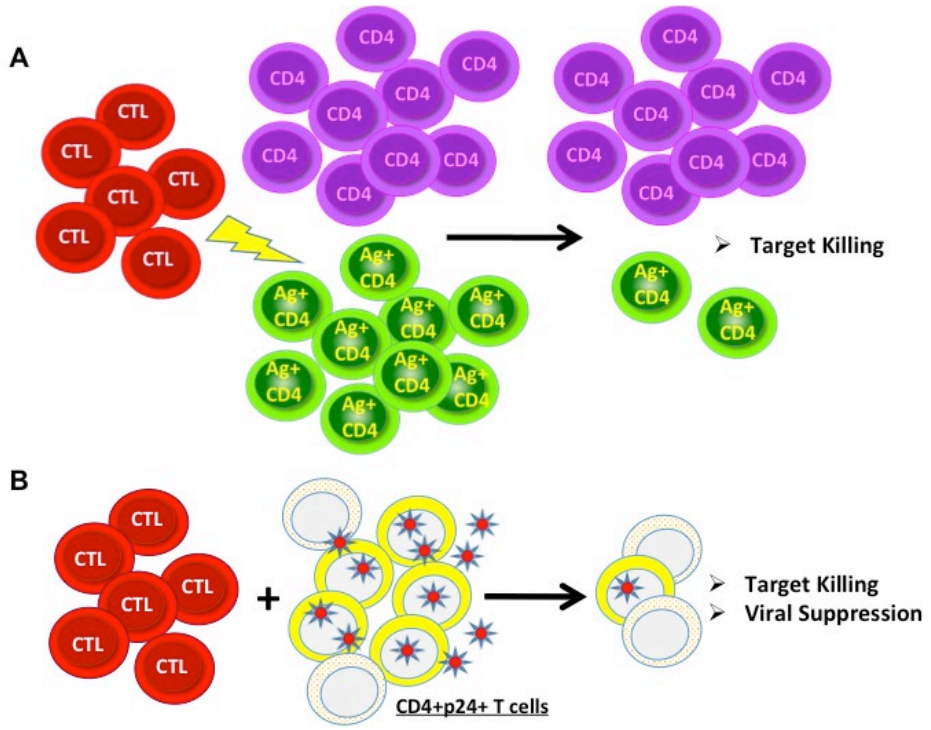


Figure 13. Model of kill assays for determination of antigen-specific elimination of the latent reservoir by autologous CTL

A) CD4$^+$ T cells were labeled with CellTrace™ dye (violet) or CFSE (green) for use as target cells. CFSE-labeled cells were additionally loaded with relevant HIV-1 Gag 9-mer peptides. Cells were mixed in equal numbers and coincubated for 18 hours with MDC1-induced autologous CTL at various E:T ratios. Antigen-specific killing of HIV-1 peptide-loaded CD4$^+$ T cells (green) was calculated based on relative changes in the percentages of viable differentially colored target cells. **B)** HIV-1 latency reversal was induced by MDC1 and SEB or antigen in CD4$^+$ T cells. Target cells (T) were coincubated with autologous MDC1-induced effector CTL (E) at various E:T ratios for 18 hours to assess target killing or 8 days to assess suppression of virus production. CTL-induced target killing was measured by loss of HIV-1 Gag p24-expressing target cells using flow cytometry. CTL-induced viral suppression was measured by reduction in culture supernatant p24 content by ELISA.

The antigen-specific killing capacity of these CTL was initially tested by coculturing them overnight with differentially labeled Gag 9-mer peptide antigen-loaded (CFSE-labeled, green) or antigen-negative/control (CellTrace™ violet-labeled) autologous $CD4^+$ T cell targets. Selective elimination of the antigen-loaded target cells was evident by flow cytometry analysis (Figure 14A, B) (424). In addition, compared to autologous HIV-specific CTL, control CTL generated using MDC1 cultured in the absence of HIV-1 Gag peptides or with irrelevant control peptides (Influenza M1) were unable to kill exposed infected targets (Figure 14C, D) (424).

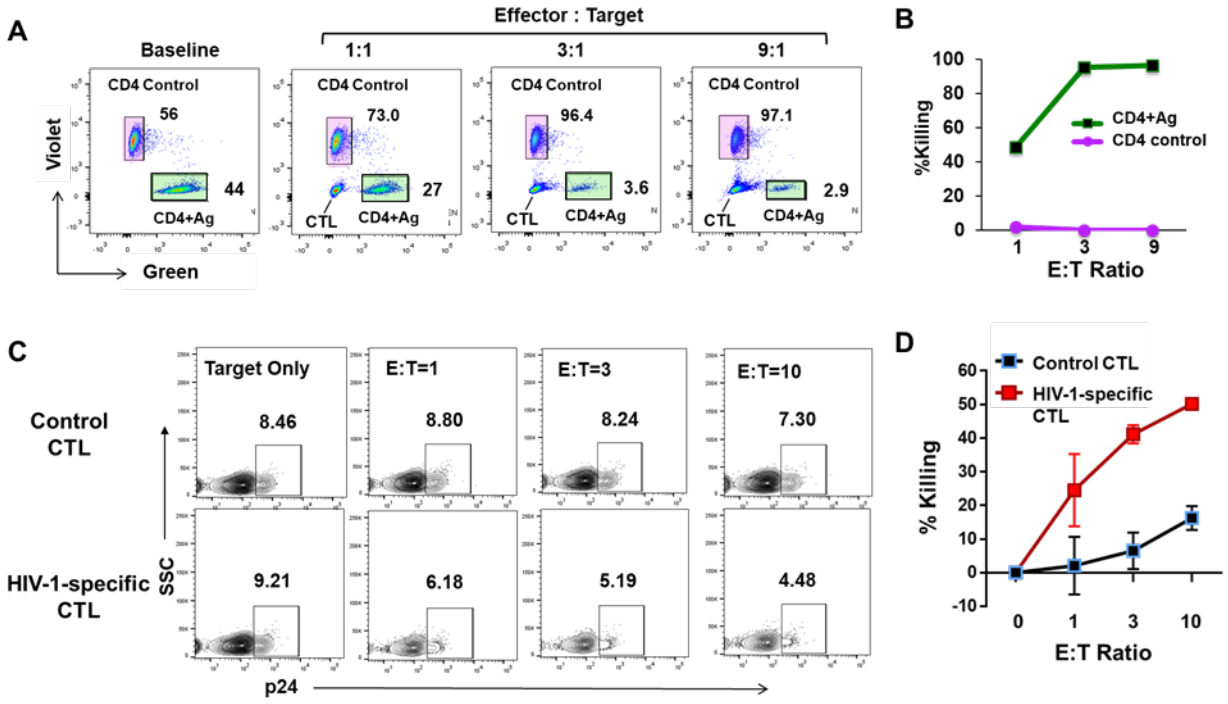


Figure 14. Selective killing of HIV-1 antigen-expressing CD4$^+$T cells by autologous MDC1-induced HIV-specific CTL

MDC1 generated from HIV-1-infected, ART-suppressed individuals were cultured with or without HIV-1 Gag peptides or with irrelevant control peptides (Influenza M1) and used to induce autologous CTL. **A)** CD4$^+$ T cells were labeled with CellTrace™ dye (violet) or CFSE (green) for use as target cells. The CFSE-labeled cells were additionally loaded with relevant HIV-1 Gag 9-mer peptides. Cells were mixed in equal numbers and coincubated for 18 hours with MDC1-induced autologous CTL at various E:T ratios. **B)** Antigen-specific killing of HIV-1 peptide-loaded CD4$^+$ T cells (green) was calculated based on relative changes in the percentages of viable differentially colored target cells. **C)** p24-expressing HIV-1-infected target cells (T) were coincubated with autologous MDC1-induced effector CTL at various E:T ratios for 18 hours. CTL-induced target killing was measured by loss of HIV-1 Gag p24-expressing target cells using flow cytometry. **D)** Comparison of cytotoxic activity of HIV-specific CTL with CTL cultured in the absence of HIV-1 Gag peptides (Control CTL) in a representative donor (shown in A). Error bars indicate mean ± S.D.

More importantly, the LR activity of CMV and HIV-1 antigen-presenting MDC1 resulted in the effective exposure of HIV-1-infected target cells that were also recognized and efficiently controlled by MDC1/Ag-induced HIV-1-specific CTL in short-term cytotoxicity assays (75.4% ± 14.3% killing), indicated by a dose-dependent decrease in p24-expressing $CD4^+$ T cells (Figure 15A, B) (424). This pattern was consistent, regardless of the type of antigen used to induce MDC1-mediated LR.

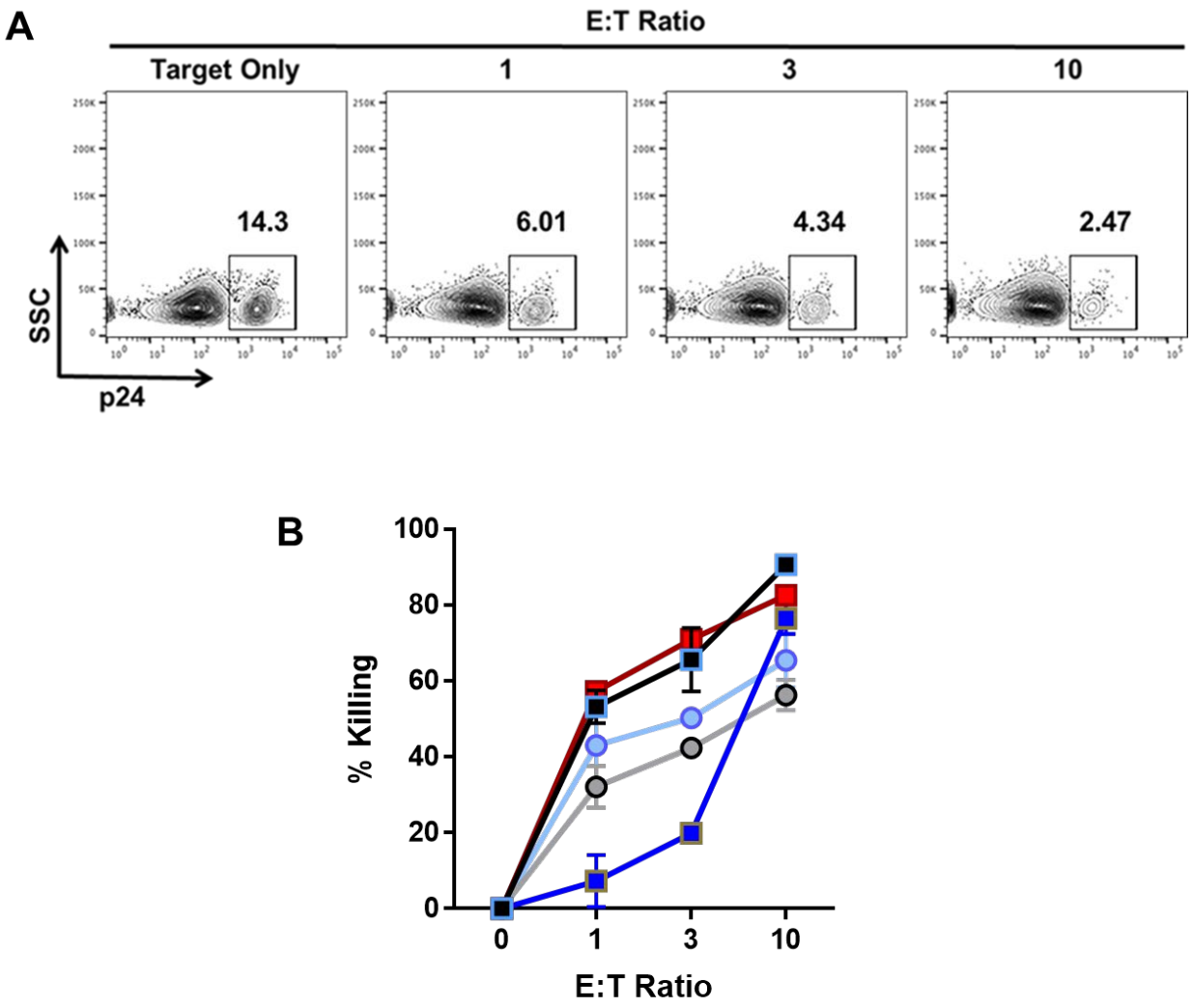


Figure 15. MDC1-induced HIV-1-specific CTL effectively kill CD4$^+$ T cells exposed by MDC1

A) HIV-1 latency reversal was induced by MDC1 and SEB or antigen in CD4$^+$ T cells. Target cells (T) were coincubated with autologous MDC1-induced effector CTL (E) at various E:T ratios for 18 hours. CTL-induced target killing was measured by loss of HIV-1 Gag p24-expressing target cells using flow cytometry. **B)** Summary of 5 independent flow cytometry cytotoxicity experiments. Square symbols represent MDC1/SEB-induced target cells; circles indicate MDC1/viral antigen-induced targets. Error bars indicate mean ± S.D.

Furthermore, CTL suppressed viral outgrowth from the infected cells in long-term cocultures, as indicated by a 74.4% ± 20.2% decrease in culture supernatant p24 (Figure 16) (424).

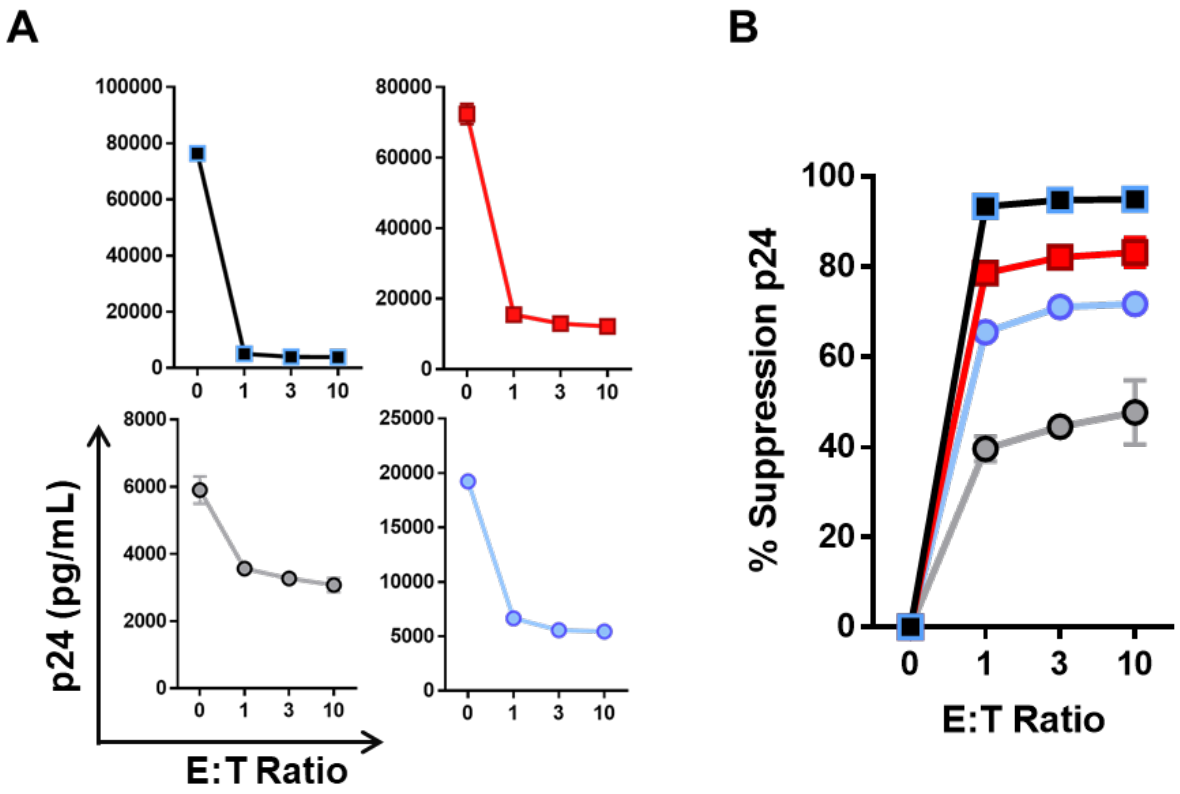


Figure 16. HIV-1-specific CTL control viral outgrowth from HIV-1-infected CD4$^+$ T cells

A) HIV-1 latency reversal was induced in CD4$^+$ T cells by MDC1 presenting SEB, CMV pp65, HIV-1 Gag, or influenza M1 antigen. Target cells (T) were cocultured with autologous MDC1-induced effector CTL (E) at various E:T ratios for 8 days. Culture supernatants were tested by p24 ELISA to measure CTL-induced viral suppression. Square symbols represent MDC1/SEB-induced target cells; circles indicate MDC1/viral antigen-induced targets. **B)** Graphical compilation of individual experiments. Error bars indicate mean ± S.D.

Recent studies have posited that CTL preferentially target cells containing defective HIV-1 proviruses, which in effect act as decoy targets to prevent elimination of the true latent reservoir (59, 295, 296). Therefore, we sought to determine whether MDC1-mediated LR activity unmasked cells harboring replication-competent virus that could be subsequently recognized and killed by the CTL. To do so, culture supernatants collected from viral outgrowth assays at various effector-to-target ratios (Figure 16) were subsequently cultured on TZM-bl reporter cells (72) for quantification of infectious HIV-1 (Figure 17). Importantly, we found that MDC1 LR activity exposed those targets harboring replication-competent virus, whose elimination was apparent at even the lowest effector-to-target ratio (Figure 17A), resulting in 84.5% ± 6.7% suppression of replication-competent HIV-1 (Figure 17B) (424). Thus, MDC1-primed HIV-1-specific autologous CTL were capable of eliminating HIV-1-infected cells harboring replication-competent virus following their subsequent unveiling through the LR activity of MHC-class II antigen-presenting MDC1.

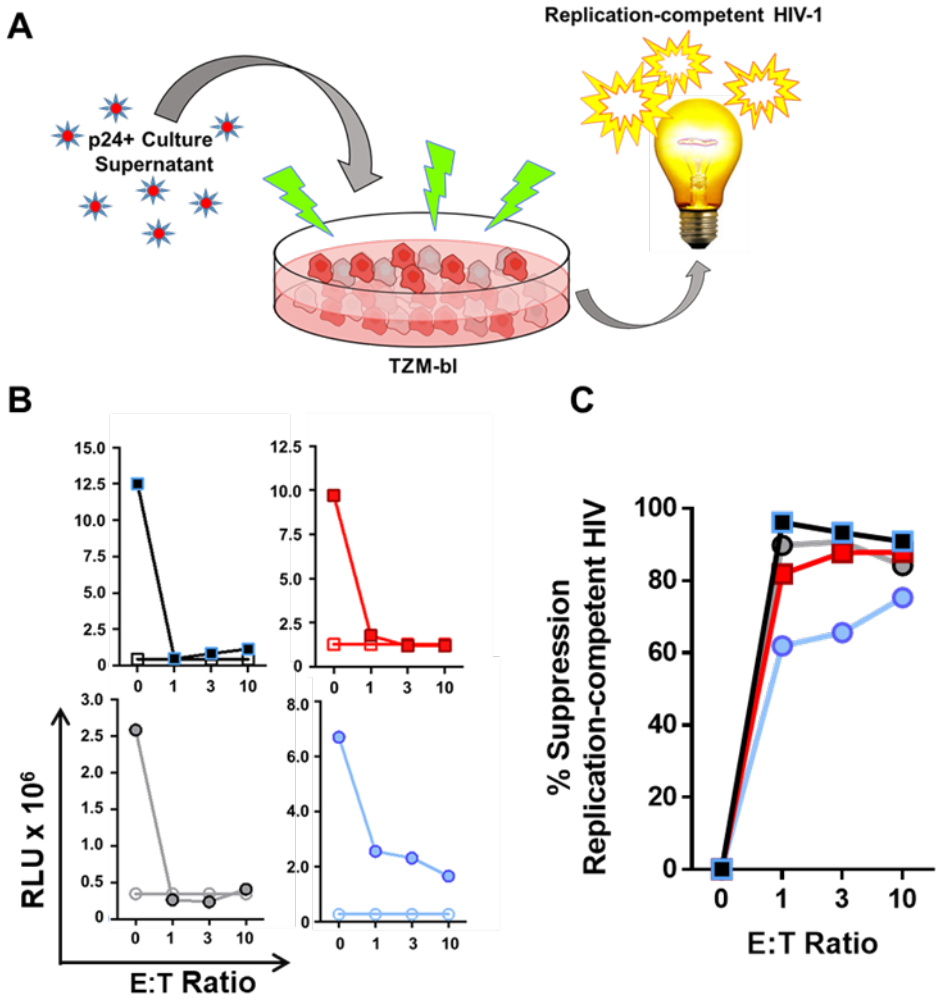


Figure 17. HIV-1-specific CTL control MDC1-exposed targets harboring replication-competent HIV-1

A) Model of TZM-bl assay for detection of replication-competent HIV-1. Culture supernatants collected from viral outgrowth assays (Figure 16) were spinoculated onto TZM-bl cell monolayers, cultured for 48 hours, and tested for chemiluminescence. **B)** Graphical compilation of individual experiments (RLU, relative light units). Solid symbols indicate HIV-1-infected participant samples; open symbols represent HIV-1-negative samples assayed in parallel. Square symbols represent MDC1/SEB-induced target cells; circles indicate MDC1/viral peptide antigen-induced targets. **C)** CTL-induced suppression of replication-competent HIV-1. Square symbols represent MDC1/SEB-induced target cells; circles indicate MDC1/viral peptide antigen-induced targets.

5.0 DISCUSSION

We have shown that antigen-presenting MDC1 are capable of inducing both HIV-1 latency reversal in infected $CD4^+$ T cells isolated from ART-treated HIV-1^+ MACS participants, and HIV-1 antigen-specific CTL responses that can effectively kill the MDC1-exposed HIV-1-infected targets (Figure 18) (424). This MDC1-mediated 'kick' was found to be antigen-dependent, and bidirectional signaling events between the MDC1 and $CD4^+$ T cells involving the CD40/CD40L signaling pathway contributed to this process (424). Other studies have explored the LRA potential of immature DC using *in vitro* models of HIV-1 latency with infected immortalized cell lines (448, 452), through *in vitro* establishment of HIV-1 latency in primary $CD4^+$ T cells of uninfected donors (447), or by addressing their nonspecific impact on *in vitro* pre-expanded polyclonal-activated T cells (446). However, to our knowledge the present study is the first to demonstrate, in a natural setting of chronic HIV-1 infection, the effective, clinically relevant use of autologous mature DC that are specifically programmed to both mediate *ex vivo* LR in freshly isolated $CD4^+$ T cells derived from individuals undergoing successful ART, and to induce effector cells capable of recognizing and eliminating the infected cells (424). Our current data imply that a component of the HIV-1 reservoir is contained within the pool of both CMV- and HIV-1-specific $CD4^+$ T cells. Importantly, our study was limited to only a small number of viral antigen sources, and to only one target protein antigen for each of the respective viruses tested. Therefore, the levels of LR induced most likely underrepresent the magnitude of HIV-1 reactivation possible if the

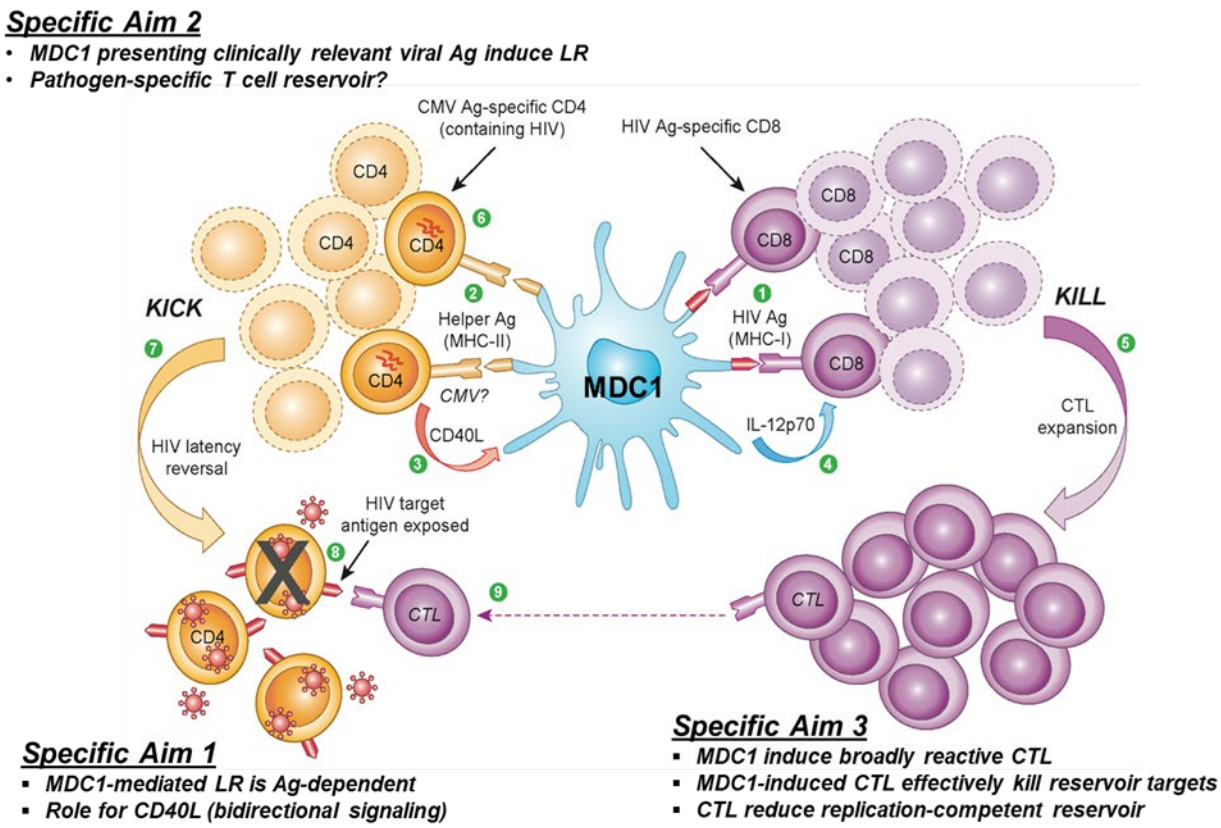


Figure 18. MDC1: the all-in-one 'kick and kill' tool

MDC1 induce antigen-specific $CD8^+$ and $CD4^+$ T cell responses through presentation of antigenic peptides in the context of **(1)** MHC class-I and **(2)** MHC class-II molecules, respectively (signal 1) , along with costimulatory factors including CD80 and CD86 (not shown, signal 2). Responding $CD4^+$ T cells subsequently provide MDC1 with the feedback hyperactivating 'helper' signal CD40L **(3)**, necessary for MDC1 release of IL-12p70 **(4)**, which then promotes expansion and differentiation of $CD8^+$ HIV-1-specific effector CTL **(5)**. Activation of CMV and HIV antigen-responsive $CD4^+$ T cells harboring latent HIV-1 **(6)** results in HIV latency reversal **(7)**, with HIV-1 proteins being transcribed and expressed as surface antigen **(8)**. As a result, exposed infected cells harboring replication-competent provirus are targeted for elimination by HIV-specific CTL **(9)**.

number and selection of antigens had been optimized. This is especially true when considering antigen-specific CMV immunity, where $CD4^+$ T cell responses to pp65 and IE-1 protein antigens comprise less than 12% of total $CD4^+$ T cell responses to CMV in coinfected individuals (502). As CMV is one of the largest and most complex viruses, with a genome encoding over 200 open reading frames (504), our study has room for optimization through incorporation of other CMV antigens that might enhance the effectiveness of our MDC1-based LR strategy. Nevertheless, our study demonstrates, as an important proof of principle, the potential that HIV-1 LR can be achieved to expose cells infected with replication-competent virus for CTL elimination, in both a safe and directed antigen-specific fashion.

Previous studies in HIV-1-/CMV-coinfected individuals indicate that HIV-1-specific $CD4^+$ T cells are preferentially infected and depleted by HIV-1 (499, 505), and that a portion of latently infected cells that remain during ART are indeed HIV-1-specific (498). However, in contrast to our findings, it has been reported that CMV-specific $CD4^+$ T cells are less susceptible to HIV-1 infection *in vivo* (506). In spite of this, a large body of data exists to support the notion that CMV antigen-specific $CD4^+$ T cells, in particular, likely contribute to a sizeable portion of the latent HIV-1 cellular reservoir. For example, CMV infection is frequent in HIV-1-infected individuals, with a seroprevalence of approximately 95% (500). Furthermore, CMV occupies an inflated proportion, on average 10%, of memory T cell responses in healthy individuals, and CMV-specific $CD4^+$ T cells persist at high levels in HIV-1- and CMV-coinfected individuals (501, 504, 507, 508), with greater than 1 out of 4 of the total number of $CD4^+$ T cells in peripheral blood being CMV-specific in some individuals (502). Subclinical CMV replication often occurs in the mucosal and peripheral tissues of HIV-1-infected individuals, contributing to T cell dysfunction, impaired immune recovery, and chronic immune activation during ART (509-512). In these environments,

CMV hijacks human cytokine/chemokine signaling to enhance inflammation, in turn augmenting its own replication (513). Consequently, recruitment of target cells to sites of inflammation that are also major sites of HIV-1 persistence, such as gut and other lymphoid tissues, creates a favorable environment for reservoir seeding (514-517). Recent evidence supporting this scenario was provided by a study of ART-suppressed individuals in whom CMV replication in the gut was associated with inflammation, mucosal barrier damage, and microbial translocation (514). Manipulation of HIV-1 coreceptor expression by CMV could also serve as a mechanism for establishment of the latent reservoir in susceptible target cells. For example, CMV upregulates CCR5 expression on newborn umbilical cord blood central memory $CD4^+$ T cells that could facilitate *in utero* transmission of HIV-1 (518). In addition, *in vitro* studies demonstrate the ability of CMV to manipulate AP-1 and NF-κB signaling for induction of HIV-1 gene expression in infected bystander cells through direct transactivation of the HIV-1 LTR (519-522). Each of these mechanisms could potentially lead to enhanced HIV-1 infection of target cells in CMV-coinfected individuals.

Once established, the latent HIV-1 cellular reservoir could also be subject to CMV-mediated proliferation or clonal expansion. Proliferation of HIV-infected cells could occur in response to persistent CMV-derived antigenic stimulation, potentially leading to compartmentalization of HIV provirus within CMV-specific $CD4^+$ T cells (516). In support of this theory, cross-sectional studies have shown a correlation between CMV replication in blood and semen and higher levels of HIV-1 DNA in both ART-naïve and ART-suppressed individuals (523, 524). In a related longitudinal study, CMV replication in PBMC of men initiating early ART was associated with delayed decay of HIV-1 DNA reservoirs (517). Furthermore, proviral and integration site analyses in ART-suppressed individuals have implicated clonal expansion of

latently infected $CD4^+$ T cells as a major mechanism of HIV-1 persistence (25, 37, 41, 47, 525), and recent findings estimate that these expanded clones comprise 50-60% of the latent HIV-1 reservoir (38, 60, 63). Of note, in a study of 15 HIV-1-infected patients who underwent myeloablative chemotherapy for CMV- and Epstein-Barr virus (EBV)-associated malignancies, increases in HIV-1 DNA were preferentially found in CMV- and EBV-specific $CD4^+$ T cells after immune reconstitution (526). It is also possible that self-renewal of stem cell memory T cells (T_{SCM}) may contribute to homeostatic proliferation of the latent HIV reservoir (527). This subset of memory T cells plays a significant role in the maintenance of long-term immunological memory and contains the most copies of integrated provirus per cell in HIV-1-infected individuals (527). Finally, CMV-infected individuals possess functional CMV-specific T_{SCM} cells that could promote expansion of the HIV-1 reservoir in CMV/HIV-1-coinfected persons through homeostatic proliferation, even during ART (528).

Coincidentally, expanded CMV-specific $CD4^+$ T cells harboring latent HIV could be protected from elimination as a consequence of numerous immune evasion strategies that CMV has evolved. CMV can not only inhibit cell surface HLA expression, but also produces decoy viral homologues of HLA class I molecules which protect it from NK cell immunosurveillance (529, 530). Similarly, CMV hijacking of cell signaling pathways could also benefit HIV persistence in coinfected individuals. Among these, IL-10 signaling is known to dampen proinflammatory immune responses and to promote a tolerogenic environment (531). IL-10, which can be induced by HIV itself, has also been shown to inhibit HIV replication and latency reversal *in vitro* (532-534). Importantly, CMV encodes viral homologues of human IL-10 (hIL-10) that produce the same immunosuppressive effects, which are further intensified by CMV's ability to upregulate hIL-10 expression during infection (535, 536). Furthermore, CMV-mediated

manipulation of PD-1 signaling may play a role in HIV-1 persistence during coinfection. PD-1 signaling on newly HIV-infected $CD4^+$ T cells has been proposed as a mechanism by which these cells transition toward a state of persistence to escape activation-induced cell death (314, 537). In support of this theory, PD-1-expressing $CD4^+$ T cells contain high levels of proviral DNA, and PD-1 expression is positively correlated with plasma viremia (314, 538). Relevant to these findings, a recent study of ART-suppressed men demonstrated an association between seminal CMV shedding and increased PD-1 expression on $CD4^+$ T cells (512). Finally, CMV could interfere with innate cellular sensors that function to trigger apoptosis of HIV-infected cells before viral integration (539, 540). CMV encodes two proteins that block apoptosis through either inhibition of cytochrome C release from mitochondrial membranes or interference with activation of caspase-8 (541, 542). In these ways, CMV could contribute to enhanced seeding of the latent reservoir during acute HIV infection (516). Thus, mounting evidence suggests that CMV coinfection could contribute to establishment and maintenance of the HIV-1 reservoir.

Conversely, Casazza *et al.* cite several factors that render CMV-specific $CD4^+$ T cells less susceptible to HIV infection (506). Based on previous findings in coinfected individuals that HIV-specific $CD4^+$ T cells are preferentially infected and depleted by HIV, CMV-specific $CD4^+$ T cells are often present in late stage HIV infection, and CMV-related disease does not occur until end stage AIDS, the group postulates that CMV-specific $CD4^+$ T cells are protected from HIV infection *in vivo* (499, 505, 543-545). The proposed mechanism underlying this protection involves increased autocrine production of β-chemokines MIP-1α and MIP-1β by CMV-specific $CD4^+$ T cells upon stimulation by cognate antigen (506). Binding of β-chemokines or RANTES to CCR5 blocks HIV infection of CMV-specific $CD4^+$ T cells by either steric hindrance of CD4 receptor binding or downregulation of CCR5 coreceptor expression (546-549). Based on their finding that

HIV infection of CMV-specific $CD4^+$ T cells was relatively low compared to infection of tetanus toxoid- and *C. albicans*-specific cells despite neutralization of β-chemokines, Hu *et al.* suggested postentry restriction as a potential mechanism for resistance of CMV-specific cells to HIV infection (550). Corroborating microarray data revealed selective upregulation of type I IFN response genes, as well as TRIM5 and TRIM22 restriction factors in CMV-specific $CD4^+$ T cells. In addition, unlike HIV-specific $CD4^+$ T cells, more mature CMV-specific $CD4^+$ T cells exhibit a phenotype of replicative senescence and therefore do not enter the cell cycle, which also limits HIV replication (196, 551-555).

Although our study points to CMV- and HIV-1-specific $CD4^+$ T cells as harboring latent provirus, it is likely that $CD4^+$ T cells specific to other viruses that manifest as chronic infections, including EBV and herpes simplex virus, contribute to the latent reservoir as well. Given the relevance of the B cell follicle as a major tissue reservoir of HIV, and that EBV is B cell-tropic, we initially considered implementing EBV antigen in MDC1-mediated latency reversal experiments in Aim 2. However, due to the aforementioned limitations of $CD4^+$ T cell recovery from our chronically infected study participants, we had to restrict our study to the use of CMV, influenza, and HIV-1 antigens. Moreover, while we did not observe influenza antigen-mediated LR *ex vivo*, others have documented increases in cell-associated HIV-1 RNA expression in HIV-1-infected individuals receiving influenza vaccination during suppressive ART (556, 557). However, deep sequencing studies pre- and post-vaccination suggested nonselective induction of proviral expression from a broad pool of HIV-1-infected bystander cells (557). While the LR demonstrated in our study was antigen-driven, we do not rule out the possibility that proviral reactivation could reflect nonspecific bystander effects of a potent MDC1-mediated antigen-specific response, rather than a direct impact on an antigen-specific reservoir.

Recent *in vivo* studies utilizing TLR7 and TLR9 agonists to promote HIV-1 LR have shown promise (192-194), and are currently being studied in human trials (http://www.clinical trials.gov NCT02858401, NCT03060447, NCT03837756). However, the mechanisms of how these innate immune receptor activators lead to reactivation of the latent reservoir have yet to be fully elucidated. Evidence from human and nonhuman primate studies implicates the IFN-α-producing plasmacytoid DC (pDC) as being an important cellular component in this process (191, 192, 194). It is worth noting that the combination of factors used in the maturation and generation process of the specialized antigen-presenting type-1 programmed MDC1 used in our study, which includes IFN-α, IFN-γ, and the TLR3 agonist poly (I:C), was designed to mimic maturation events expected to occur as a result of DC crosstalk with responding IFN-α-producing pDC and IFN-γ-producing NK cells during the early stages of a successful antiviral immune response (383, 407). The factors produced by these early immune responders programs the maturing DC to be hyper-responsive to subsequent signaling factors they receive during antigen cognate interactions with $CD4^+$ T_H cells.

Although the underlying mechanisms of MDC1-mediated LR observed in our study have not been fully identified, our findings do indicate that bidirectional cross-talk between the MDC1 and the $CD4^+$ T cells during cognate antigen-driven interaction involving the CD40/CD40L signaling pathway contributed to the noted LR activity (424). Based on gene chip analysis of CD40L-activated MDC1 that revealed upregulations in galectin-9, TNF-α, and IL-15 mRNA (data not shown), all of which have been implicated as potential inducers of LR (212, 221, 446), these factors warrant further investigation.

Furthermore, targeting the true latent reservoir poses numerous challenges with regard to antigen delivery by a DC vaccine *in vivo* to certain sites that are anatomical sanctuaries of HIV-1, such as B cell follicles (322). It is well documented that secondary lymphoid organs (SLOs) such

as the spleen, lymph nodes, and gut-associated lymphoid tissue (GALT) are the major sites of HIV replication. B cell follicles found within SLOs contain numerous phenotypically and functionally distinct cell types. Whereas $CTLA4^{+}CD4^{+}$ T cells are the primary targets of HIV infection within the T cell zone of lymph nodes (558), the T follicular helper (T_{FH}) and T follicular regulatory (T_{FR}) subsets that reside within the B cell follicle are not only more susceptible to infection (317-319), but harbor the highest concentration of HIV-1 RNA in both untreated chronic SIV and HIV infection prior to the development of AIDS (321, 559, 560). Indeed, follicular $CD4^{+}$ T cells are 30-40 times more likely to contain HIV RNA than extrafollicular (EF) $CD4^{+}$ T cells (320, 321). The T_{FH} subset responsible for germinal center (GC) reactions, affinity maturation, class switching, and memory B cell differentiation (561) is most prevalent within the B cell follicle (324). *In vivo* infection of T_{FH} cells may be influenced by their close spatial relationship with follicular dendritic cells (FDC) carrying HIV immune complexes (IC), which accounts for their increased susceptibility to FDC-bound IC-mediated infection compared to EF $CD4^{+}$ T cells (562). T_{FH} cells are further subdivided into germinal center T_{FH} (GC-T_{FH}) and non-germinal center T_{FH} (non-GC T_{FH}) subsets expressing high and intermediate-to-low levels of PD-1, respectively. The $PD\text{-}1^{hi}$ GC T_{FH} cell subset is more susceptible to infection, and thus GCs contain the greatest concentration of HIV RNA+ cells within the B cell follicle (318). Interestingly, $PD\text{-}1^{+}$ memory T_{FH} were recently reported to be the major reservoir of replication-competent provirus in ART-suppressed individuals (55). However, compared to EF, T_{FH}, and EF T_{REG} subsets, the highest percentage of HIV RNA+ cells is found within the recently identified T_{FR} subset (319, 563-565). This population modulates T_{FH} functions to prevent autoimmunity (565), but HIV-induced T_{FR} expansion and increases in expression of regulatory factors by T_{FR} enhance this effect, potentially contributing to T_{FH} dysfunction during chronic infection (566). Notably, changes in the frequency

and function of T_{FH} and T_{FR} that impact somatic hypermutation are thought to contribute to the inability of most HIV-infected individuals to produce bNAb (567, 568).

Despite decreases in the frequency of follicular HIV RNA+ cells during ART, these cells are still detectable (118, 569, 570). Furthermore, HIV-IC bound to the surface of FDC present in LN, GALT, and spleens constitute a major reservoir (571-573) of long-lived, infectious virus even in the presence of neutralizing antibodies (562, 574, 575). Importantly, infected EF $CD4^+$ T cells are subject to CTL-mediated elimination, but latently infected cells within the follicle are protected from CTL control, as most CTL do not express the follicular homing receptor CXCR5 (320). In addition to limitations of trafficking, some reports suggest that follicular $CXCR5^+CCR7^-$ $CD8^+$ T cells are less cytolytic than $CXCR5^-CCR7^-$ extrafollicular CTL, expressing regulatory or exhausted phenotypes, lower levels of perforin, and decreased polyfunctional cytokine responses (311, 325, 326). Conversely, other studies document that although less frequent, the $CXCR5^+CD8^+$ T cell population exhibits increased cytolytic potential compared to $CXCR5^-CD8^+$ T cells, including higher levels of perforin and IFN-γ production and a less exhausted phenotype (311, 327). Thus, the mechanisms governing CTL trafficking and cytolytic activity within B cell follicles are largely unknown. Novel strategies to address barriers to CTL targeting of this compartment are being explored, including temporary disruption of the B cell follicle with depleting antibodies (anti-CD20/rituximab), blockade of T and B cell interactions using anti-CD40L, and therapeutic vaccination with engineered CTL or chimeric antigen receptor T cells expressing CXCR5 (54, 324). Treatment with recombinant IL-15 or the IL-15 superagonist ALT-803 has also been proposed as a method of enhancing follicular CTL function, as both have been shown to augment HIV-specific CTL responses in addition to their latency reversal properties (212, 324). Furthermore, ALT-803 has advanced to a clinical trial in ART-treated HIV-infected individuals

(http://www.clinical trials.gov NCT02191098). Another elegant strategy involves the use of bispecific antibodies targeting CD3 and HIV gp120 that have the dual capacity to act as LRAs and to facilitate ADCC in gp120-expressing cells, as well as induce killing of HIV-infected cells by follicular CTL (311, 330). As such, lymph node-homing $CXCR5^{+}CD8^{+}$ T cells could be transduced to produce these antibodies in order to facilitate killing of latently infected cells within the B cell follicle (324). Though not addressed in this study, the question of whether MDC1 express CXCR5 is clinically relevant to latency reversal approaches targeting the B cell follicle and warrants investigation. MDC1 could also be transduced to express CXCR5 to facilitate follicular homing *in vivo*. In addition to these strategies, the LRA potential of antigen-presenting B cells or other immune cells should be explored as potential components of a targeted immunotherapy. Inextricably linked, all of these factors highlight the significance of the B cell follicle in HIV persistence and necessitate its careful consideration in HIV cure strategies. Nonetheless, the results presented here provide strong rationale for the incorporation of MDC1 in a dual therapeutic approach for both the 'kick' and the 'kill' of latent HIV-1.

6.0 IMPLICATIONS TO PUBLIC HEALTH

In December 2013, the UNAIDS 90-90-90 initiative set ambitious targets to scale up global HIV treatment beyond 2015 as a call to end the AIDS epidemic by 2030 (576). This three-part target proposed that by 2020, 1) 90% of all individuals living with HIV will be diagnosed, 2) 90% of all people diagnosed with HIV will receive sustained antiretroviral therapy, and 3) 90% of all ART-treated individuals will achieve viral suppression (576). At the end of 2017, 75% of people living with HIV knew their status, 79% of those diagnosed were receiving ART, and 81% of individuals accessing treatment had achieved viral suppression (577). Despite these successes, coverage gaps in treatment and resources still affect entire regions and populations. Progress in meeting the 2020 targets is falling behind in western, central, and North Africa; eastern Europe, central Asia, and the Middle East (577). Among factors contributing to this lack of progress is the increased risk of HIV infection among young women in areas of high HIV prevalence, such as Africa and the Caribbean (3). In addition, barriers to traditional HIV testing services continue to impact adolescents, men, and key populations, including people who inject drugs, men who have sex with men, sex workers, transgender people, and prisoners (3). Lack of political commitment, stigma and discrimination, HIV criminalization, and lack of access to affordable care are major factors undermining progress toward the 90-90-90 targets (3, 577). Thus, given the logistical and economic challenges in delivering optimal, lifelong treatment to the more than 35 million people living with HIV, there is heightened interest in finding an effective method of controlling the virus in the absence of ART (187). According to the goals set forth in the International AIDS Society global scientific strategy of 2016, development of a safe, affordable, and scalable HIV cure strategy is paramount (187).

Despite recent cases of what were initially considered functional HIV-1 cures, to date only one case of complete viral eradication in a patient, a 'sterilizing' cure (578), has been recorded. Also known as the 'Berlin patient', Timothy Ray Brown is an HIV-positive individual who underwent stem cell transplantation during chronic infection for acute myelogenous leukemia in 2007 (579). The stem cell donor was homozygous for the CCR5Δ32 mutation (580), which results in a truncated, nonfunctional variant of the CCR5 coreceptor, rendering carriers resisitant to CCR5-tropic HIV infection. Five years post-transplant, neither HIV-1 RNA nor DNA could be detected in PBMC, lymph node, spinal fluid, or terminal ileum in the absence of ART (83). In addition, Brown's antibody and T cell responses to the virus had diminished, and replication-competent HIV could not be cultured from PBMC (83). However, plasma HIV RNA and rectal HIV DNA could be detected, albeit at levels lower than those typically observed in ART-suppressed individuals, and were considered probable false positive results. Though Brown is deemed to be clinically cured of HIV-1 infection, detection of residual viremia may also reflect persistent HIV, highlighting the need for more sensitive methods to quantify the latent reservoir. Nevertheless, the recent report of the 'London patient' provides new evidence that complete eradication of persistent HIV may be achievable (581). As of March 2019, the HIV-positive man remains in HIV remission in the absence of ART, 19 months after receiving a bone marrow transplant for Hodgkin's lymphoma from a CCR5-negative donor.

Viral rebound in the absence of ART represents HIV replication from stable reservoirs (17), and trials implementing analytic treatment interruption (ATI) have revealed a correlation between total proviral DNA and time to viral rebound (582, 583). Hence, current HIV-1 eradication studies focus on the timing of viral rebound following ART cessation as a measure of reservoir reduction (83, 87, 579, 584). Several cases of long-term control of HIV-1 replication in

the absence of ART, or 'functional' cures (578, 585), have been reported. The 'Boston patients' underwent allogeneic stem cell transplants during ART, one for treatment of Hodgkin's lymphoma and the second for diffuse B cell lymphoma and subsequent Hodgkin's lymphoma (586). Unlike the cases of Timothy Ray Brown and the 'London patient', stem cells were instead obtained from HIV-1-susceptible CCR5 wild-type donors (586). Although virus was undetectable in peripheral blood and gut-associated lymphoid tissue (GALT) for years post-transplant and during ART, both chronically HIV-infected individuals experienced rebound viremia within 3-8 months of ATI, as measured by plasma HIV-1 RNA and cell-associated HIV-1 DNA (586). In agreement with previous studies (582, 583), Henrich *et al.* suggested long-lived tissue reservoirs may have facilitated viral persistence and subsequent lack of virological control upon ART cessation in these patients.

Results of the VISCONTI Study showed that ART initiation in HIV-infected individuals during acute infection decreased reservoir seeding in long-lived resting $CD4^+$ T cells (587). These post-treatment controllers (PTCs) lacked protective HLA alleles characteristic of elite controllers and strong HIV-specific $CD8^+$ T cell responses, but were somehow able to maintain control of viremia for several years following ART interruption (587).

Another example illustrating the impact of early ART on reservoir size and viral rebound is the case of the 'Mississippi baby' (588). Born to an untreated HIV-infected mother, the infant received ART 30 hours after birth due to high-risk exposure. Infection was confirmed by repeated detection of HIV RNA and DNA, and ART was administered until discontinued by the child's mother after 18 months. Although viremia remained below the level of detection for over two years following treatment interruption, viremic relapse occurred at 27 months (589, 590). As observed in the 'Boston patients', lack of HIV-1-specific immune responses combined with the

presence of replication-competent virus in the 'Mississippi baby' confirms that a latent reservoir still persists in these individuals despite early ART (17).

A final documented case of a functional HIV-1 cure is that of a perinatally infected child who received treatment early and remained in virological remission for over 12 years after ART interruption (591). Although the infant received HIV prophylaxis for six weeks after birth, HIV DNA was detected at four weeks of age, prompting the initiation of ART at 3 months. Suppressive ART was discontinued by the child's family at approximately six years of age, and HIV RNA levels below the limit of detection, accompanied by stable CD4 counts, were maintained for over 11 years (591). Interestingly, the adolescent also exhibited characteristics similar to those reported in adult PTCs, including suboptimal HIV-specific $CD8^+$ T cell responses (591).

Due to the relative success of combination antiretroviral therapy (cART) since its advent in 1996 (4), HIV infection is now managed as a chronic condition. Accordingly, one might argue that pursuit of a sterilizing, or even a functional HIV-1 cure is no longer warranted. However, while current treatment regimens are more tolerable and less toxic (228), perturbations in lipid and glucose metabolism have been observed in individuals on long-term ART (592). These metabolic imbalances manifest in numerous comorbidities that impact this aging population, including cardiovascular and liver disease, diabetes, and cancer (593-596). Contributing to these conditions are residual immune activation and inflammation that persist even in individuals on successful ART, especially in those who fail to restore $CD4^+$ T cell counts (597-599). HIV-related systemic immune activation has been linked to immunosenescence, which also predisposes infected individuals to non-AIDS-defining comorbidities such as atherosclerosis and cardiovascular disease, neurodegeneration, and cancer (600). Although ART has reduced the risk of death from HIV-1 infection, infected individuals are at greater risk of morbidity and mortality as a result of

these non-AIDS-related conditions than the general population (601-603). Drug toxicities, the potential for developing drug resistant virus, and treatment adherence issues are additional challenges associated with lifelong ART (187). All of these factors underscore the need to explore therapeutic alternatives that involve either durable control of viremia in the absence of ART or complete eradication of viral reservoirs.

Findings of the current study have the potential to transform the functional HIV cure approach through implementation of a precise, DC-based immunotherapy that elicits potent effector responses to autologous viral antigens while exposing the HIV cellular reservoir for immune targeting by promoting antigen-driven latency reversal in responsive HIV-infected $CD4^+$ T cells. To date, the majority of 'shock and kill' strategies have focused on reactivation of proviral DNA using pharmacological LRAs, none of which have achieved reductions in the latent reservoir in over 15 completed clinical trials (121, 122). HIV eradication trials have also revealed that the 'kill' does not passively follow the 'shock' induced by current LRAs (122, 287), in part due to the numerous immunosuppressive effects of various LRAs on CTL function (159, 226, 297, 468, 470). These include increases in $CD8^+$ T cell death and exhaustion marker expression, as well as decreases in CD3 expression and $CD8^+$ T cell proliferation, IFN-γ production, and killing capacity (159, 226, 297). Furthermore, HDAC inhibitors also negatively impact NK cell function (298). In contrast, DC-based therapies have proven to be safe (443) and well tolerated in clinical settings, and would serve as a more natural way to activate the HIV cellular reservoir. To date, one of the most impressive HIV immunotherapy trials, reported by Garcia *et al.* (427), utilized DC pulsed with inactivated autologous HIV, which resulted in a significant decrease in HIV RNA setpoint and was associated with increased anti-HIV $CD8^+$ T cell responses. However, as with many other DC-based studies, including those previously used by our group (426, 442), the Garcia trial

implemented DC generation methods that yield IL-12p70-deficient DC (383, 385). In an attempt to address this, Argos Therapeutics used a DC-based approach involving *ex vivo* genetic manipulation of DC via electroporation to deliver a constitutive CD40L helper signal to the DC, which failed as an immunotherapy for early and chronic HIV infections (435-437). Perhaps explaining these results, we have shown that constitutive CD40L signaling results in a burst of IL-12p70 production *in vitro*, but ultimately leads to IL-12p70-exhausted, T_H cell-unresponsive DC (294). Importantly, results from a clinical trial by our group unexpectedly demonstrated that delivery of a DC-based therapeutic HIV vaccine using inactivated autologous HIV as antigen was associated with unprecedented, increased viremia in ART-suppressed individuals (442). However, because that clinical study was also not designed to address the use of DC as a therapeutic LRA, a number of questions regarding the roles of DC polarization and antigen presentation in *in vivo* LR were left unanswered.

Because of their recognized superior potential to drive long-lived CTL responses from naïve T cell precursors (457, 458), the specialized type-1 polarized DC-based therapeutic platform (MDC1) used in the current study (424) has been successfully implemented with impressive results in cancer trials (368, 396, 429, 454-456) and is currently in early stages of FDA-approved phase I HIV clinical trials in Thailand (RV490/DC-03/SEARCH033) and Pittsburgh (DC-04) cohorts. This strategy exploits DC programming for timely production of IL-12p70 upon subsequent antigen-specific interaction with $CD4^+$ T_H cells to drive type-1 polarized immune responses (383, 385, 410). Both studies are designed to target only highly conserved HIV-1 epitopes using MDC1 loaded with conserved HIV-1 peptide libraries as the vaccine, but differ from one another by studying acute (Thailand) and chronic (Pittsburgh) ART cohorts. Findings of the current study have been incorporated in a grant proposal to enhance the study design of an already approved

MDC1-based clinical HIV trial in Brazil, setting it apart from the aforementioned MDC1 trials by adding CMV-associated MHC-class II peptide antigen as an immunotherapeutic. Current and proposed trial arms are detailed in Figure 19. The primary aims of the original phase I clinical study were to assess the safety and tolerability of an αDC1 (MDC1) therapy in participants receiving early ART (Fiebig 1-5), evaluate virologic and immunologic impacts of the αDC1 therapy, and correlate these findings with alterations in GALT. Primary endpoints of the original and proposed studies include safety and changes in plasma viral load, and $CD4^+$ T cell setpoints following analytic treatment interruption (ATI). The three arms of the original trial included 1) placebo control, 2) αDC1 loaded with inactivated autologous HIV, and 3) αDC1 loaded with inactivated autologous HIV, followed by temporary ATI. The modified study employing CMV antigen will consist of 4 treatment arms: 1) αDC1 empty control; 2) αDC1 loaded with inactivated autologous HIV; 3) αDC1 loaded with CMV peptide; 4) αDC1 loaded with inactivated autologous HIV and CMV peptides. All proposed treatment groups will include 10 participants per group receiving three rounds of subcutaneous αDC1 injections followed by ATI.

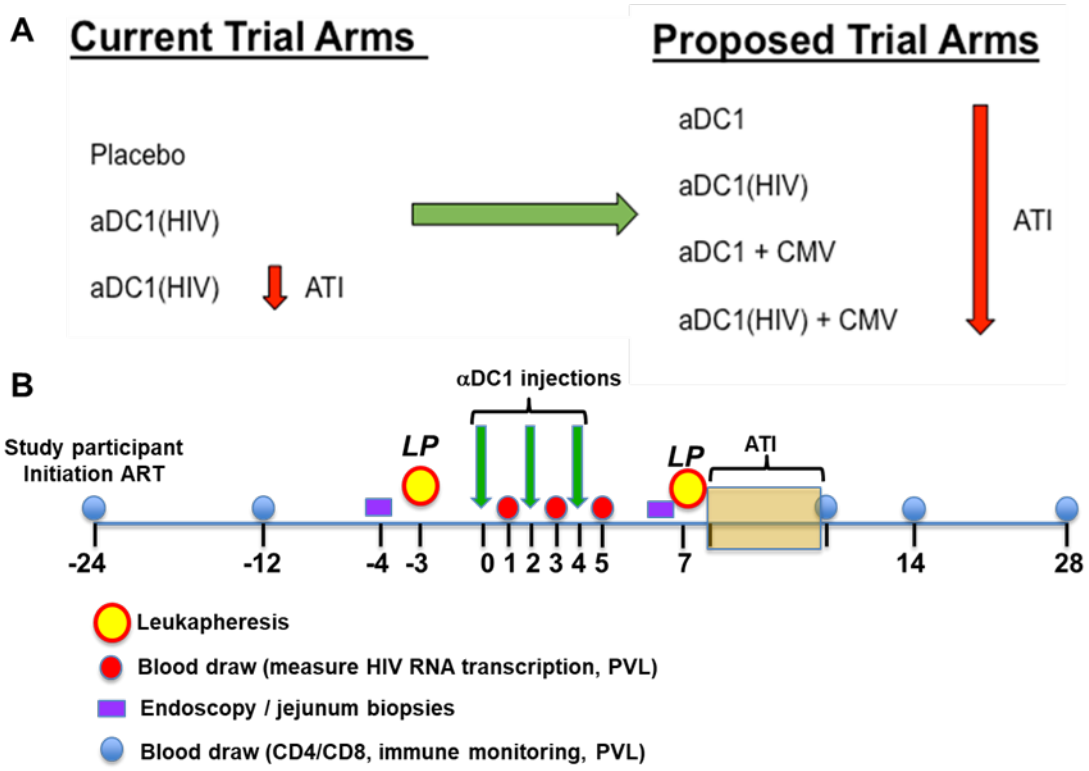


Figure 19. Brazil/USA αDC1-based HIV-1 immunotherapy trial

A) Treatment groups of original (left) and proposed (right) αDC1 clinical trial. **B)** Timeline of study, therapy regimen, and sample collection schematic for the study participants; PVL, plasma viral load.

Importantly, our study supports the concept that CMV-specific $CD4^+$ T cells could harbor a prominent portion of the HIV latent reservoir. We hypothesize that this is based on persistent, low-level reactivation of latent CMV and consequent stimulation of CMV-specific, $CD4^+$ memory T cells that become HIV-infected and preferentially survive as a quiescent HIV DNA reservoir. Thus, the use of CMV antigen in the proposed strategy can serve two important functions. First, for the HIV 'kill' it will promote MDC1 interaction with $CD4^+$ T cells that can provide the CD40L 'helper' activation signal required to promote MDC1 IL-12p70 production and induction of long-

lived primary HIV-1-specific CTL responses (294, 383, 486-488, 604, 605). Second, for the HIV 'kick', this approach can facilitate the targeted unveiling of responsive CMV antigen-specific $CD4^+$ T cells, which themselves could represent a large portion of the latent HIV reservoir. As other CMV vaccine preparations are currently in clinical trials in HIV-negative individuals, we believe that a CMV peptide-based vaccine is a highly immunogenic formulation that is both safe and currently acceptable for human clinical use (606-608). In the Brazilian cohort, this unique approach will test the dual capacity of the MDC1 therapeutic to facilitate HIV latency reversal and expose the viral reservoir, while concurrently boosting cellular immune effectors to kill the HIV-infected cells. Thus, information gained from these studies could lead to the durable control or cure of HIV-1 infection.

Nevertheless, due to the numerous stakeholders in cure research, overcoming the challenges associated with either complete eradication of HIV or a functional cure will require multidisciplinary global collaboration that extends beyond biomedical research (187). Due to the complexity and rapid evolution of cure science, research on science translation and public engagement will be critical to the success of cure strategies (187). First, the perceptions of people living with HIV must be central in the design of cure approaches. Serostatus is often a factor in decisions regarding the health and psychological well-being of these individuals, influencing not only sexual behaviors but social involvement (187). As a result, their conception of HIV cure may directly influence their trust and engagement in HIV services and health care, willingness to disclose serostatus, and risk behaviors (187). As demonstrated by examples of fraudulent and ineffective HIV 'cures' in sub-Saharan Africa (609), these beliefs could also determine the willingness of people living with HIV to participate in clinical trials and shape their perceptions of the benefits and risks of cure research (187). Thus, research into the ethical, personal,

behavioral, and social implications of participation in cure research, as well as effective communication of the science and associated risks and benefits of HIV immunotherapies, must be a focus of cure strategies moving forward (187).

Second, early engagement of stakeholders at multiple levels and standardized tools for measuring stakeholder involvement must be implemented to foster the success of cure efforts (610). Stakeholders in cure research include but are not limited to individuals living with HIV, health care professionals and scientists, funding and regulatory agencies, public health authorities, civil society organizations, and pharmaceutical companies (187). Collaboration between these diverse groups can also help prevent or mitigate potential failures of cure strategies (610).

Equally important to the future success of HIV cure efforts is the equitable distribution of therapies among diverse trial participants in terms of sex, age, ethnicity, and other demographic characteristics (187). Moreover, modeling research could provide scientists with information to help optimize clinical trials by predicting which individual or combination of cure approaches has the potential to achieve population-level effects (611). In this regard, both affordability and cost-effectiveness will be deciding factors, especially in resource-limited countries that have disproportionately high incidences of HIV infection and would benefit the most from a cure (612). Finally, determining the cost of an HIV cure and assigning responsibility for payment will be crucial considerations in the design of feasible interventions (187). Despite all of these challenges, the ever-changing landscape of HIV research has produced promising therapeutic strategies that have the potential to cure a disease once considered a death sentence. However, this goal will never be attainable as a result of scientific discovery alone, but only in conjunction with identification and elimination of the social and economic barriers that pose an equal threat to an HIV cure.

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